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**14. ABSTRACT**

Breast cancer is the most common type of cancer and a leading cause of death among Western women. Most breast cancers (75%) express ERα, and antiestrogens have been widely used in their treatment. However, acquired resistance and unwanted side effects in other estrogen-responsive tissue such as uterus have greatly limited their use. The objective of this study to isolate RNA aptamers that specifically target the estrogen receptor interacting NR boxes/LxxLL motifs of the MED1 protein and test their efficacy on breast cancer cell growth both in vitro and in vivo by utilizing a pRNA nanodelivery system for tissue-selective therapy. In this funding period, we have generated large amount of highly purified MED1 protein and RNA aptamer library. We further carried out and optimized SELEX experiments to select RNA aptamers that specifically bind wild type but not mutant MED1 LxxLL motifs. After 6 rounds of selections, we have obtained and tested 11 RNA aptamers. With our establishment of RNA nanoparticle system, we are now ready to further test these RNA aptamers on ER-mediated functions and breast tumor growth both in vitro and in vivo.

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## Table of Contents

## Introduction

Breast cancer is the most common type of cancer and a leading cause of death among Western women. Most breast cancers (75%) express ERα, and antiestrogens have been widely used in their treatment. However, acquired resistance and unwanted side effects in other estrogen-responsive tissue such as the uterus have greatly limited their use. This study is based on our recent unexpected finding that disruption of the transcriptional coactivator MED1 nuclear receptor-interacting NR boxes/LxxLL motifs in vivo specifically impaired ERα function in pubertal mammary gland development, but did not affect the development of other estrogen-responsive tissues. Significantly, MED1 is reported to be overexpressed and amplified in a high proportion of primary breast cancers and breast cancer cell lines, which has recently been confirmed in several genome-wide microarray analyses of human breast cancer patient samples. We *hypothesize* that targeting MED1 NR boxes /LxxLL motifs by aptamers will lead to tissue-selective blockage of the estrogen signaling pathway and inhibition of human breast cancer cell growth. The objective of this study is to isolate RNA aptamers that specifically bind the MED1 LxxLL motifs and test their efficacy on breast cancer cell growth both in vitro and in vivo by utilizing a pRNA nanodelivery system.

## Body

To achieve our goals above, we have generated MED1 NR boxes expression vector and subsequently purified wild type and MED1 NR boxes /LxxLL motifs mutant protein. In addition, we have also constructed RNA aptamer library and worked out the SELEX (Systematic Evolution of Ligands by Exponential Enrichment) procedure to isolate RNA aptamers that bind to MED1 NR boxes/LxxLL motifs. Building upon these, we have now successfully carried out 6 rounds of SELEX procedures and obtained top RNA Aptamer candidates that interact with MED1 NR boxes during this funding period. We have further tested their binding capacity and ability to disrupt ER/MED1 interactions both in vitro and in breast cancer cells and made significant advancement in the following areas of the study:

### SELEX Experiments

As mentioned in last report period, we have purified approximately a total of 10mg of NR Box proteins through FPLC and 1mg of RNA library for the SELEX experiment. After 5 rounds of SELEX procedures, the recovery rate of the RNA bound to our target protein from the SELEX experiment has increased 256 fold. We then cloned the resulting RT-PCR fragments of this round into Topo TA cloning vector. We eventually sequenced 50

| | RNA Library | Protein | Bound RNA | Recovery Rate |
|---|---|---|---|---|
| **Round 1** | 499.9 ug | 1mg | 65ng | 0.013% |
| **Round 2** | 131ug | 1mg | 35ng | 0.026% |
| **Round 3** | 500.42ug | 1mg | 310ng | 0.06% |
| **Round 4** | 119ug | 1mg | 1075ng | 0.90% |
| **Round 5** | 300ug | 300ug | 2990ng | 3.32% |
| **Round 6** | 236.8ug | 300ug | 3185ng | 4.48% |

**Table I. RNA Recovery Rate after each round of SELEX procedure.** The table shows the amount of RNA library, protein used in each round of SELEX procedure and the bound RNA recovered. The data indicate an about 345 fold increase of RNA recovery rate after 6 rounds of SELEX procedures.

clones and obtained the DNA sequence coding for these RNA aptamers. After analyses of the sequence obtained, we found there are a total of 7 sequences that showed up multiple times, including 4 that showed up 3 times. To ensure the success of SELEX experiments, we further carried out an additional round of SELEX procedure with both positive and negative selections using wild type and mutant MED1 NR box proteins. As shown in table I, the RNA recovery rate was able to increase a little bit further although not as dramatic as previous rounds. Overall, after six total rounds of SELEX, the recovery rate of total RNA had increased ~345 fold. For this sixth round of RNA recovered, we sent both the before SELEX and after SELEX samples for the next generation sequencing (RNA-seq). Through this, we obtained a total of ~12,000,000 sequence reads. The abundance of each aptamer sequence varies from a single copy to up to 1M copies. It is also worth noting that many of the top candidates from this round were also present in the previous round. Based on the sequencing results from both rounds, we eventually selected a total of 11 top candidates for further testing as described below. For each of the aptamers selected, we also assigned a random name, which in most cases codes for a color designated.

**Binding Test of Selected Individual Aptamers**

We next carried out binding assays to further test the binding of these individual aptamers to MED1 NR Box proteins. In these assays, we not only included wild type MED1 NR box protein but also mutant MED1 NR box proteins with mutations on NR box A, or B, or Both. The experiments were set up as follows: 1) His-tagged NR-Box wild type protein, incubated with control scramble aptamer or each of the selected aptamers; 2) His-tagged double mutant protein, incubated with control scramble aptamer or each of the selected aptamers 3) His-tagged NR-Box single mutant A protein, incubated with control scramble aptamer or each of the selected aptamers; and 4) His-tagged NR-Box single mutant B protein, incubated with control scramble aptamer or each of the selected aptamers. After incubation, the samples were then spun down by centrifugation,

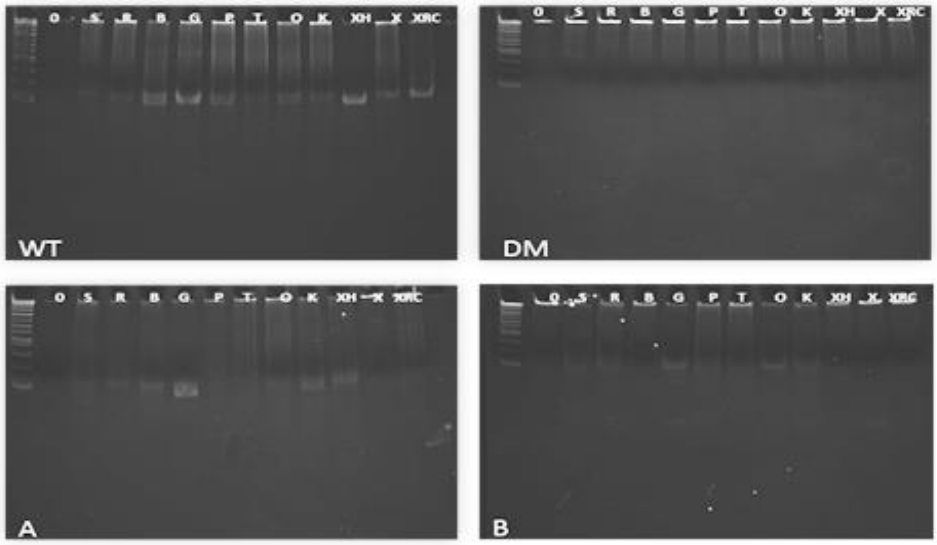


**Fig 1. Binding of the selected RNA Aptamers to wild type and mutant forms of the MED1 NR-Boxes.** Control scramble and top 11 RNA aptamers candidates obtained from the SELEX experiments were mixed with indicated MED1 NR box wild type and mutant proteins. After extensive wash, bound RNAs were subjected to RT-PCR and native PAGE gel analyses. WT indicates the wild type form of NR-Box. DM indicates the double mutant form (LXXAA, LXXAA), A indicates the LXXAA mutant in the first motif and B indicates the LXXAA mutant in the second motif. The data indicate that these isolated aptamers bind to WT but not DM MED1 NR boxes as expected. Interestingly, they also exhibited different characteristics in their binding preferences to these MED1 NR boxes.

followed by phenol chloroform/ chloroform extractions to separate the bound aptamers from the protein. After ethanol/NaAC precipitations, the RNA was reverse transcribed and amplified by PCR, and then run through an 8% Native PAGE gel. As shown in Fig. 1, we observed specific binding of wild type NR-Box proteins to each of the RNA aptamers we isolated but not control scramble, with some aptamers showing a higher binding affinity than others. Importantly, none of these aptamers showed binding to mutant MED1 NR box protein, suggesting that these NR boxes are required for their interactions. For the A and B MED1 NR box mutants, we found some aptamers exhibited a higher specific affinity for the first LXXLL (mutant B) motif than the second, as seen with RNA aptamers P and O. In contrast, some aptamers showed a higher affinity for the second LXXLL motif (mutant A) as demonstrated by aptamers B,G, K and XH. Interestingly, the rest of aptamers, in contrast, show no preference of either LXXLL motif, as demonstrated by similar band strength between A and B gels for aptamers R, T, X and XRC. All together, we confirmed that the RNA aptamers we isolated can bind to MED1 NR boxes while at the same time they also exhibited differential binding preferences and requirement for these two LXXLL motifs.

**GST-Pulldown Assay**

To further test whether these RNA aptamers are able to disrupt the interactions between ER and MED1 NR-Box, we carried out GST-Pull down assays using these individual RNA aptamers using purified GST-ER and MED1 NR-Box proteins. For each setup, these 3 components were incubated with each other, pulled down by GST beads through centrifugation, and subjected to western blot analyses after separation by SDS/PAGE gel. Negative controls in this experiment consisted of unbound GST incubated with WT NR-BOX, shown in the left-most lane below in Fig. 2, along with GST-bound ER, shown next to the GST + NR-Box WT lane. Positive controls consisted of GST-bound ER (GST-ER) incubated with WT NR-Box and then

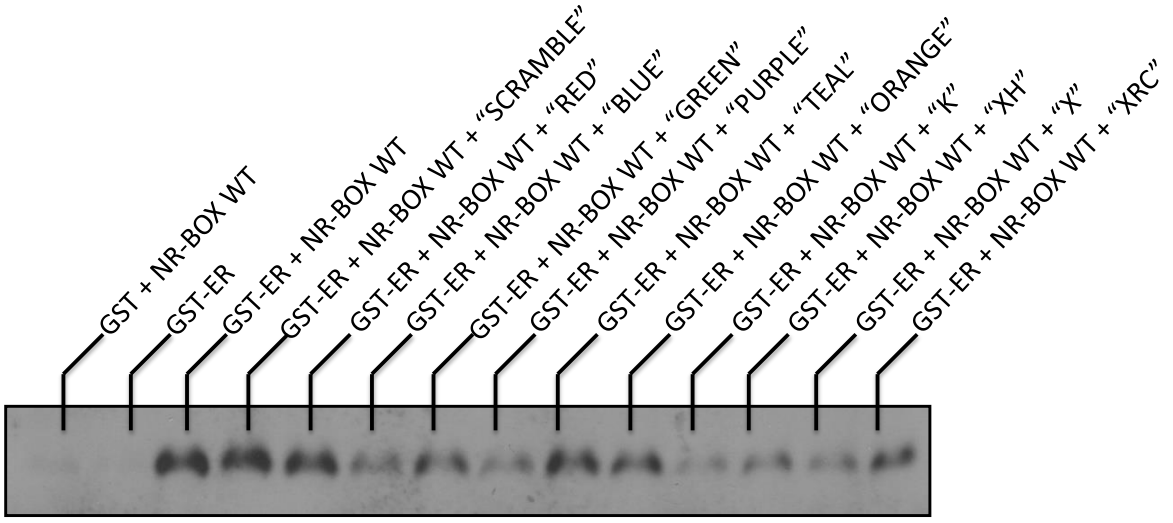


**Fig. 2. Disruption of the interaction between wild type NR-Box and Estrogen Receptor by selected RNA Aptamers.** GST-pulldown assays were carried out using GST-ER LBD (ligand binding domain) and MED1 NR-Box fragment in the presence of estrogen and the indicated RNA aptamers. Western blot analyses were carried out to detect the bound MED1 NR boxe protein. The data indicate the several RNA aptamers we isolated were able to disrupt the interaction between ER and MED1 NR box to various degrees in this in vitro binding assays.

this same set up with a random "scramble" aptamer composed of a completely unselective sequence. The positive controls are shown directly to the right of the negative controls on the figure below. By comparing these controls alone, we can see that our pull-down is working efficiently because without both ER and WT NR-Box, nothing will be pulled down as expected. We can also see that the randomly chosen RNA aptamer has no effect on the interaction between ER and NR-Box, as indicated by the "scramble" control lane. Importantly, after comparing the effect of each of the selected aptamers to this "scramble" control aptamer, we observed inhibitory effects of most of these selected RNA aptamers on the interactions between ER and MED1 NR-Box proteins to some degrees. Of particular interest are aptamers B (Blue), G (Green), P (Purple), K, XH and X as these aptamers showed a very significant reduction in ER/MED1 NR-Box interaction as indicated by the much lighter bands observed in the blot.

Effects of RNA aptamers on ER-Mediated Transcription

To move a step further of above in vitro binding and interaction assays, we next examined whether these RNA aptamers will affect ER-mediated transcription in vivo in cultured cells. Specifically, we preformed ERE (estrogen-responsive elements)-Luciferase reporter assays to determine the effect of these aptamers on estrogen-dependent reporter gene expression activities. We first cultured ER positive human breast cancer MCF-7 cells in phenol-free medium and seeded them in 24 well plates. ERE luciferase reporter and pRL-CMV, a Renilla control reporter, were then transfected into the cells, along with a different RNA aptamer for each column of wells. Approximately 4-6 hours after transfection, cells were induced with either

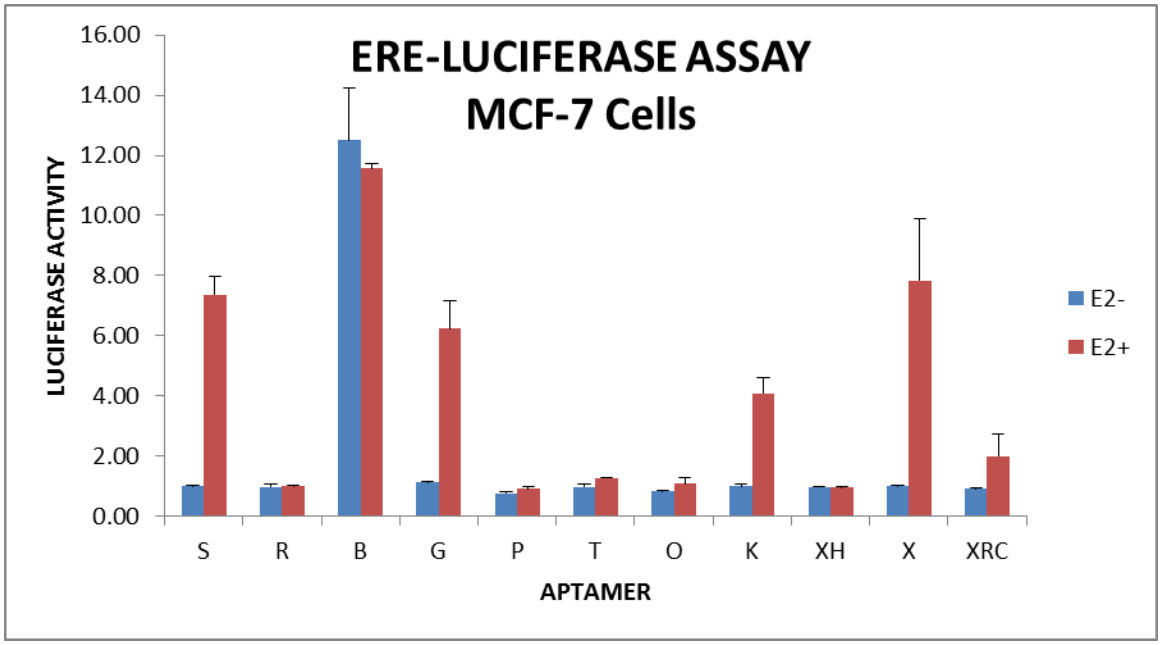


**Fig. 3. Inhibition of ERE-luc activities in MCF-7 cells by selected RNA Aptamers.** Control Scramble and above mentioned RNA aptamers were co-transfected with ERE-Luciferase reporter into MCF-7 cells cultured in phenol-red free medium supplemented with charcoal stripped serum. Control vehicle or estrogen was added ~4-6 hours later for another 24-36 hrs incubation before harvesting cells for luciferase activities measurement using dual-luciferase reporter assay kits and a luminometer. The data indicate that several RNA aptamers we isolated through abvoe SELEX procedure (R, P, T, O, and XH) were able to completely block the estrogen-dependent induction of ERE-luciferase activities.

30nM beta-estradiol or control vehicle ethanol. After approximately 24-36 hours, cells were harvested and the luciferase activities were measured using a GloMax 20/20 luminometer. The data indicate that control scramble (S) aptamer did not affect the estrogen-induced activation of ERE-reporter gene expression as we were still able to achieve the optimal amount of estrogen induction (~7 fold) as we routinely obtained previously without any addition of aptamers. We then compared the luciferase activities of our selected RNA aptamers with this scramble control. Of interest are the R, P, T, O, XH and XRC aptamers that were able to almost completely block the estrogen-induced ERE-luciferase activities in these cells. These data further support our above in vitro studies and suggest that these aptamers we obtained may represent a new class of aptamers that can effectively inhibit transcription of ER genes mediated by the interaction of ER and NR-Box of MED1.

**RNA Nanoparticle Syntheses and its Functional Test**.

In addition, we have started to set up and establish the RNA nanoparticle synthesis and testing procedure in preparation for further in vitro and in vivo examination of aptamers isolated above on ER-mediated functions and breast cancer cell growth. To synthesize pRNA/MED1 siRNA and control pRNA/scramble siRNA, in vitro transcriptions were conducted. Briefly, we first synthesized PCR primer pairs with the 5' end primer containing the T7 phage 2.5 promoter sequence followed by the sense sequence of the desired siRNA, and the 3' end primer containing the antisense sequence of the siRNA. These specifically designed primers were then used to amplify cDNA fragments using the plasmid pRNA(A-b') DNA or nts 23/97 pRNA(A-b') DNA as templates. The amplified cDNA templates were transcribed with T7 RNA polymerase in the presence of ATP, GTP, UTP, and CTP (7.5 mM each). The final transcription products (pRNA/scramble siRNA and pRNA/MED1 siRNA) were purified and then tested for cell growth assay using MCF-7 cells (Fig. 4). MCF-7 cells were plated at 10,000 cells/well in DMEM containing 10% fetal bovine serum. Control pRNA/Scramble siRNA and two pRNA/MED1 siRNA with different siRNA sequences were introduced into these cells through lipofectamine 2000 (invitrogen). Cells were collected and counted every 24 hours for 9 days, and plotted. The data indicated successful knockdown of MED1 (Fig. 4A) and inhibition of MCF-7 cell growth by both pRNA/MED1 siRNAs while compared to that of control pRNA/scramble siRNAs (Fig. 4B). Therefore, we are in a very good position to use RNA nanoparticles system to test the functions of above isolated RNA aptamers on ER-mediated functions and breast cancer cell growth.

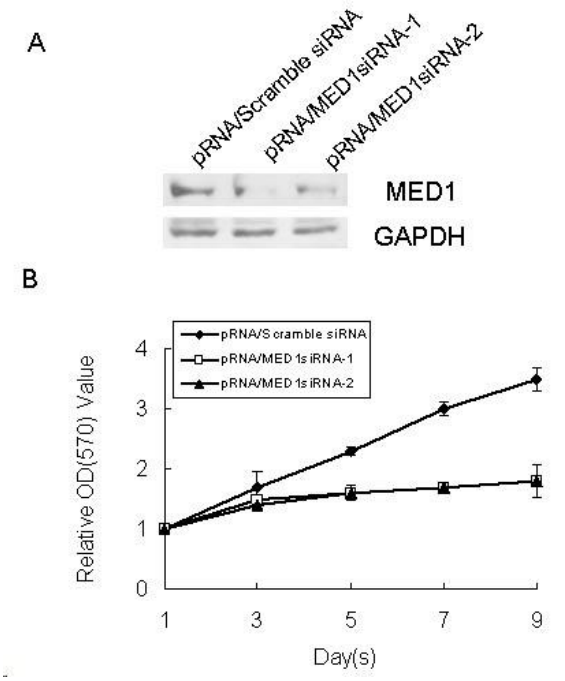


**Fig. 4: pRNA/MED1 siRNAs inhibit MCF-7 cell growth.** Western Blot (A) and cell growth (MTT) assay of MCF-7 cells after treatment with pRNA/Scramble siRNA and two independent pRNA/MED1 siRNA. The data shows that both pRNA/MED1 siRNA are able to knockdown MED1 expression and inhibit MCF-7 cell growth.

## Key Research Accomplishments

- Six rounds of SELEX have been completed, showing an overall recovery rate increase of ~345 fold.
- A total of 11 Aptamers with highest percentage out of total sample and highest frequency between multiple rounds were chosen after analysis of GENEWIZ conventional sequencing and state-of-art RNAseq data.
- Binding assays were completed to test which aptamers had highest affinities for WT NR-Box and also to test whether aptamers had different affinities for either of the two LXXLL motifs on MED1.
- Aptamers B,G,K and XH showed higher affinity for the second LXXLL motif and aptamers P and O showed higher affinity for the first LXXLL motif, while the rest of aptamers showed similar affinity between the two mutant forms, indicating that they do not have a preference for either motif.
- GST-Pulldown assays were conducted to test ability of each aptamer to block interactions between GST-bound ER and NR-Box WT
- ERE-Luciferase assays were further carried out to test which aptamers could block ER-NR-Box interaction in MCF-7 cells.
- Carried out procedures for the construction of RNA nanoparticles using pRNA and MED1 siRNA sequences and confirmed knockdown of MED1 and inhibition of MCF-7 cell growth. This prepares for our further experiments to incorporate the above isolated RNA aptamers and test their function on ER-mediated functions and breast cancer growth both in vitro and in vivo.

## Reportable Outcomes

In this reporting period, we have three manuscripts (Attached as Appendices for this report) published and one abstract accepted by international conferences:

***Papers:***

Germer K, Leonard M, and **Zhang X.** RNA aptamer and its diagnostic and therapeutic applications. (2013) Int J Biochem Mol Biol. 2013 Mar 31; 4(1):27-40.

Germer K, Pi M, Guo P and **Zhang X.** Conjugation of RNA aptamer to pRNA nanoparticles for RNA-based therapy. (2013) RNA Nanotechnology and Therapeutics, CRC Press 2013 July. Pages: 399-408

M Czyzyk-krzeska and **Zhang X**. MiR-155 at the Heart of Oncogenic Pathways. (Commentary) *Oncogene*. (2013) Advance online publication, 18 February 2013; doi:10.1038/onc.2013.26

***Abstracts:***

Bauer-Nilsen K, Leonard M, Cui J, Wu T, Wang J, Chen C, Guo P and **Zhang X**. Estrogen Receptor Coactivator MED1 in Endocrine Resistance and as a Therapeutic Target for Human Breast Cancer. 2nd International Conference of RNA Nanotechnology and Therapeutics. Apr. 4-6, 2013. Lexington, KY

## Conclusion

In conclusion, during this funding period, we have successfully completed the SELEX procedures and selected 11 top candidates based on their highest percentage of presence out of total sample and highest frequency between multiple SELEX rounds. Importantly, we have also employed several different approaches to test the binding of these aptamers to both wild type and mutant MED1 LxxLL motifs and further examine their roles in ER-mediated interactions and functions not only in vitro but also in vivo in cultured human breast cancer cells. In addition, we have also established procedures for the construction of RNA nanopartilces and successfully tested their functions in human breast cancer cells in our laboratory. Therefore, we are in a very good position to perform further experiments to incorporate the best RNA aptamers that has been further selected by our above assays into the RNA nanoparticles and test their functions on ER-mediated functions and breast cancer cell growth both in vitro and in vivo. In addition to these, we have also published one book chapter and one review article on RNA aptamers and the approach for its conjugation to RNA nanoparticles for targeted drug delivery, and presented our work at an international conference.

## References

**N/A**

## Appendices

Paper 1: Conjugation of RNA aptamer to RNA nanoparticles for targeted drug delivery.

Paper 2: RNA aptamers and their diagnostic and therapeutic applications.

Paper 3: MiR-155 at the Heart of Oncogenic Pathways.

# 19

# *Conjugation of RNA Aptamer to RNA Nanoparticles for Targeted Drug Delivery*

**Katherine Germer, Fengmei Pi, Peixuan Guo, and Xiaoting Zhang**

## CONTENTS



## 19.1 Introduction

RNA-based therapy has become a promising avenue for the treatment of many human diseases. The therapeutic potential of RNAs, including ribozymes, short hairpin RNA, small interfering RNA (siRNA), microRNA, antisense oligonucleotides, and RNA aptamers, has long been extensively studied (Guo 2010; Keefe et al. 2010; Levy-Nissenbaum et al. 2008; Que-Gewirth and Sullenger 2007; Yan and Levy 2009). A major challenge that remains is the systemic delivery of these moieties (siRNA, ribozyme, etc.) to the desired target cell organelles. In this regard, the packaging RNA (pRNA) nanoparticle delivery system pioneered by Dr. Guo, combined with recent advancement in RNA aptamers, provides an ideal method for nanoscale delivery suitable for in vivo targeted delivery (Guo 2010).

pRNA nanoparticles are a new RNA-based nanoparticle drug delivery system, which can be designed and constructed by phi29 pRNA through dimer formation, hexamer formation, or using its stable three-way junction (3WJ) domain as a scaffold. Because pRNA nanoparticles are composed entirely of RNA, it is the most natural choice for the delivery of RNA therapeutics because using an all-RNA delivery system will also allow all the advantages of RNAs as therapeutic agents to be retained. One key obstacle for RNA-based therapy is that RNA is susceptible to quick degradation in the bloodstream during in vivo delivery. To overcome this, researchers from Dr. Guo's laboratory have recently developed

highly stable and RNase-resistant pRNA through elaborate design of RNA sequences and chemical modifications such as 2′-deoxy-2′-fluoro (2′-F) modification at the ribose rings of C and U (Liu et al. 2011; Shu et al. 2011).

RNA aptamers are RNA oligonucleotides capable of binding to specific targets with high affinity and specificity. RNA aptamers have numerous advantages for targeted drug delivery when compared with DNA aptamers, protein aptamers, and antibodies (Guo 2010; Keefe et al. 2010; Que-Gewirth and Sullenger 2007; Thiel and Giangrande 2010). Compared with their peptide and antibody counterparts, RNA aptamers are much easier to synthesize in large quantities with defined structure and stoichiometry. Furthermore, RNA aptamers are generally considered to be more thermodynamically stable than peptides or antibodies. Although RNA aptamers function similarly to antibodies, they are known to have low or no immunogenicity when compared with other macromolecules such as proteins/antibodies. Furthermore, recent studies have found that RNA aptamers can be further chemically modified (e.g., 2′deoxy, 2′F, 2′NH3, 2′OMe) to achieve high stability and evade RNase shearing even in the bloodstream. Moreover, the single-stranded nature of RNA aptamers not only allows them to form unique tertiary structures for tighter and more specific binding to the target but also makes them smaller in size and thus allowing easier access into cells compared with other types of aptamers. Thus, the conjugation of RNA aptamers to delicately designed pRNA nanoparticles can assist in the delivery of therapeutics harboring RNA nanoparticles to specific cell organelles to maximize therapeutic effects while minimizing the toxicity of the drug delivery system.

## 19.2 Structure of RNA Nanoparticles

The concept of RNA nanotechnology has been proposed for more than one decade. Most RNA molecules are single-stranded nucleotides, which can adopt very complex three-dimensional structures. Therefore, RNA is an ideal biocompatible material that is suitable for construction at the nanometer scale for drug delivery, especially for oligonucleotide-based drug delivery.

### 19.2.1 RNA Nanoparticle Formed by Dimerization of phi29 pRNA

Phi29 pRNA is a 117-nucleotide bacteriophage phi29 encoded pRNA discovered by Dr. Guo in 1987 (Guo et al. 1987). The pRNA monomer plays an essential role in packaging DNA into procapsid by forming a hexamer ring to drive the DNA packaging motor of bacteriophage 29, which is approximately 11 nm in size. The primary structure of wild-type pRNA is described in Figure 19.1 (Liu et al. 2011). pRNA has two functional domains that can fold independently: the DNA translocation domain and the prohead binding domain. The DNA translocation domain is composed of a 3′/5′ double helix loop whereas the prohead binding domain is composed of left-hand and right-hand loops. If we name the right-hand loop with uppercase letters (A, B, C) and name the left-hand loop with lowercase letters (a, b, c) denoting different loop sequences, the RNA sequence for A, B, C are complementary to sequence a, b, c, respectively. pRNA dimer nanoparticles can be formed through the complementary hand-in-hand loop interactions between pRNA monomer Ab and Ba as described in Figure 19.2 (Shu et al. 2004). The pRNA dimer nanoparticle has been reported to have a particle size of approximately 25 nm (Chen et al. 2000), which allows it to be employed as a nanoparticle carrier for gene drug delivery because its small size allows it to escape from being engulfed by the reticuloendothelial system, and to be used for repeated and long-term gene drug delivery.

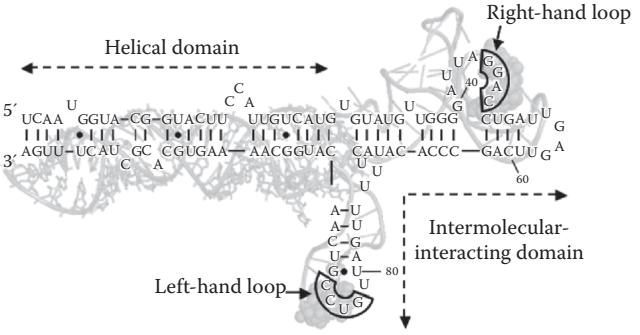


**FIGURE 19.1**
Primary sequence and structure of wild-type pRNA. (Reprinted with permission from Liu, J. et al., Fabrication of stable and RNase-resistant RNA nanoparticles active in gearing the nanomotors for viral DNA packaging, *ACS Nano* 5, 237–246. Copyright 2011 American Chemical Society.)

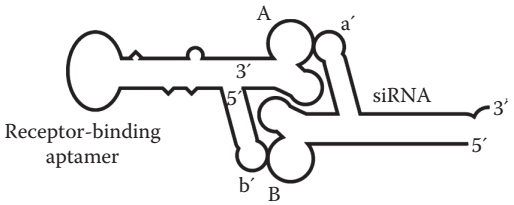


**FIGURE 19.2**
pRNA dimer formed through hand-in-hand complementary loop interactions. (Reprinted by permission from Macmillan Publishers Ltd. Guo, P. et al., The emerging field of RNA nanotechnology, *Nat Nanotechnol* 5, 833–842, copyright 2010.)

### 19.2.2 RNA Nanoparticle Composed by Using pRNA Hexamer as Scaffold

pRNA dimers are building blocks for pRNA hexamer formation, which has been proved by Chen et al. (2000), through hand-in-hand interactions between two complementary pRNAs that can form dimers, tetramers, and hexamers. A schematic drawing for the pRNA hexamer formation is described in Figure 19.3. The six pieces of pRNA in pRNA hexamer nanoparticles could provide six positions to conjugate therapeutic molecules such as siRNAs, ribozymes, therapeutic RNA/DNA aptamers, or diagnostic RNA/DNA aptamers for drug delivery.

### 19.2.3 RNA Nanoparticles Based on 3WJ Motif

A third strategy for constructing pRNA nanoparticle is based on a thermodynamically stable RNA 3WJ motif (Shu et al. 2011). pRNA has two functional domains that can fold independently: a DNA translocation domain and a prohead binding domain. The two domains are connected by a 3WJ motif, as described in Figure 19.4. The 3WJ domain of pRNA was demonstrated to be very stable, which can retain its folding even in 8 M of urea or at very dilute concentrations. By conjugating the RNA therapeutic molecules such as siRNA or RNA aptamers to the 3WJ motif, they can self-assemble to form RNA nanoparticles, which can potentially be used for targeted RNA therapeutic delivery in vivo.

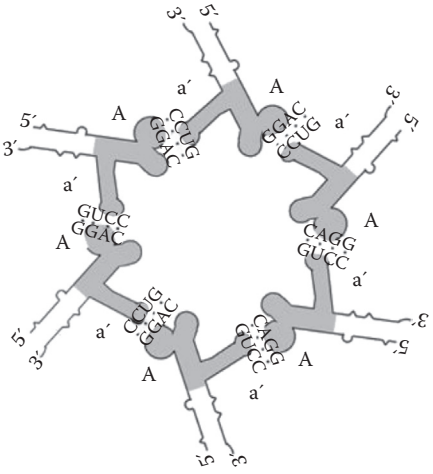

**FIGURE 19.3**
Schematic drawing of pRNA hexamer through complementary loop–loop interaction with six pieces of pRNA monomer. (From Chen C. et al., *J. Biol. Chem.* 275, 17510–17516, 2000. With permission from the American Society for Biochemistry and Molecular Biology.)

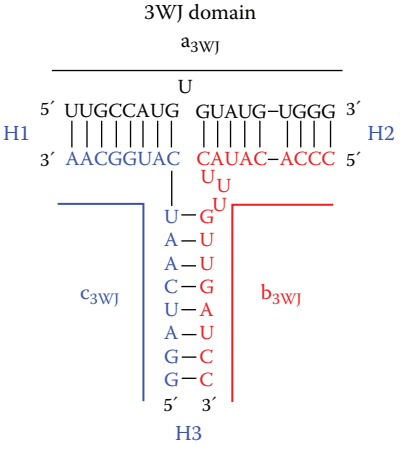


**FIGURE 19.4**
3WJ motif of pRNA, which can be used for RNA nanoparticle construction. (Reprinted by permission from Macmillan Publishers Ltd. *Nat. Nanotechnol.*, Shu, D. et al., Thermodynamically stable RNA three-way junction for constructing multifunctional nanoparticles for delivery of therapeutics, 6, 658-667, copyright 2011.)

## 19.3 Conjugate RNA Aptamer to RNA Nanoparticles for Targeted Drug Delivery

The concept of a targeted drug delivery system was proposed by Paul Ehrlich in 1902, in which he first called the hypothetical drug a "magic bullet" (Ehrlich 1957). In general, targeted drug delivery requires the targeted drug delivery system (TDDS) to selectively

deliver therapeutics to diseased regions, independent of the method of its administration. The TDDS can be classified into three grades from the aspect of the region it reaches: the first grade targeting system refers to delivering the drug to a targeted organ or tissue, the second grade targeting system refers to delivering the drug to specific cells, and the third grade targeting system refers to delivering therapeutic molecules to specific locations inside the cell.

The TDDS can also be divided into three types based on the pattern of targeting: passive, active, and physical. There has been intensive research on passive targeting drug delivery systems such as liposomes, nanoemulsions, microcapsules, and polymeric nanospheres in the last several decades. Passive targeting relies on the natural distribution pattern of the drug delivery system, as the drug carriers can be ingested by macrophages of the reticuloendothelial system and then transferred primarily to the liver and spleen. However, it is difficult to deliver drugs to other organs with the passive targeting mechanism because the in vivo distribution of the passive targeting drug carriers is greatly affected by its particle size and the surface property of nanoparticles. As a general rule, when the particle size is larger than 7 μm, it will be retained by the smallest lung capillaries through mechanical filtration; when the particle size is smaller than 7 μm, it is ingested by macrophages in the liver and spleen; carriers of particles between 200 and 400 nm are usually collected and are rapidly cleaned up by the liver. Active targeting preparations instead utilize modified drug carriers as a "bullet" to directionally concentrate drugs to the target area for enhanced efficacy. These modifications include PEGylation of the nanoparticles to conceal the particle from macrophages, conjugation with special ligands or antibodies, which can interact with the target cell receptor, and other approaches. Physical and chemical targeting preparations utilize physical or chemical properties to help navigate preparations to specific targeting locations. For example, magnetically targeted drug delivery incorporates magnetic material into the drug preparation, and the preparation will then be concentrated to the specific target area under the guidance of an externally applied magnetic field, whereas thermal or pH targeting drug delivery utilizes thermal or pH-sensitive material to deliver the therapeutics to specific macroenvironments by changes in temperature or pH.

### 19.3.1 RNA Aptamers and SELEX

RNA aptamers are RNA oligonucleotides that bind to a specific target with high affinity and specificity, similar to antibody interaction with antigens. RNA aptamer isolation was initially developed in two separate laboratories by Turek and Gold (1990), and by Ellington and Szostak (1990) through a process that eventually became known as systematic evolution of ligands by exponential enrichment, or SELEX. To begin the SELEX process, a library of randomized pools of RNA will first be synthesized. Generally, these RNA oligonucleotides are designed with a random sequence of nucleotides of approximately 20 to 80 nucleotides in the center region that is flanked on either side by a constant sequence. This library of oligonucleotides will then be exposed to the target of interest, which could be small molecules, proteins, cells, or even organisms (Dua et al. 2011; Keefe et al. 2010; Levy-Nissenbaum et al. 2008; Thiel and Giangrande 2010). Those that do not bind to the target are washed away and discarded, whereas those that do bind are isolated and amplified through reverse transcription and PCR to generate a corresponding DNA library. The DNA library is then subjected to RNA transcription, and the resulting RNA library will then be exposed again to the target of interest for another round of the SELEX process. This process is usually repeated about 5 to 15 times, and the aptamers obtained could often reach a high picomolar to low nanomolar range of dissociation constants ($K_d$) with the target (Dua et al. 2011; Yan and Levy 2009).

Through this basic SELEX process, and some recently developed variations of this process such as Cell-SELEX, Cross-over SELEX, and Tissue-SELEX, a good number of RNA aptamers have been isolated with the capability of binding numerous specific targets (Dua et al. 2011; Levy-Nissenbaum et al. 2008; Yan and Levy 2009). Significantly, many of these targets are cell surface markers of various human diseases, which has led to the application of these RNA aptamers for targeted delivery of RNA therapeutics, especially those based on RNA interferences: siRNA, short-hairpin RNA, or microRNA.

### 19.3.2 Approaches to Conjugate Aptamer to RNA Nanoparticle

The key step in the construction of RNA aptamer–conjugated nanoparticles is to design the global structure according to the physical and chemical properties of the RNA nanoparticles. If the RNA aptamer has been selected with a known sequence, it can be conjugated into the RNA nanoparticle structure before in vitro transcription or chemical synthesis of RNA. Dr. Guo and his colleagues have successfully conjugated malachite green aptamer to RNA nanoparticles characterized by a 3WJ pRNA motif. The in vitro experiment indicated that the aptamer is still functional after conjugation into 3WJ-pRNA nanoparticles

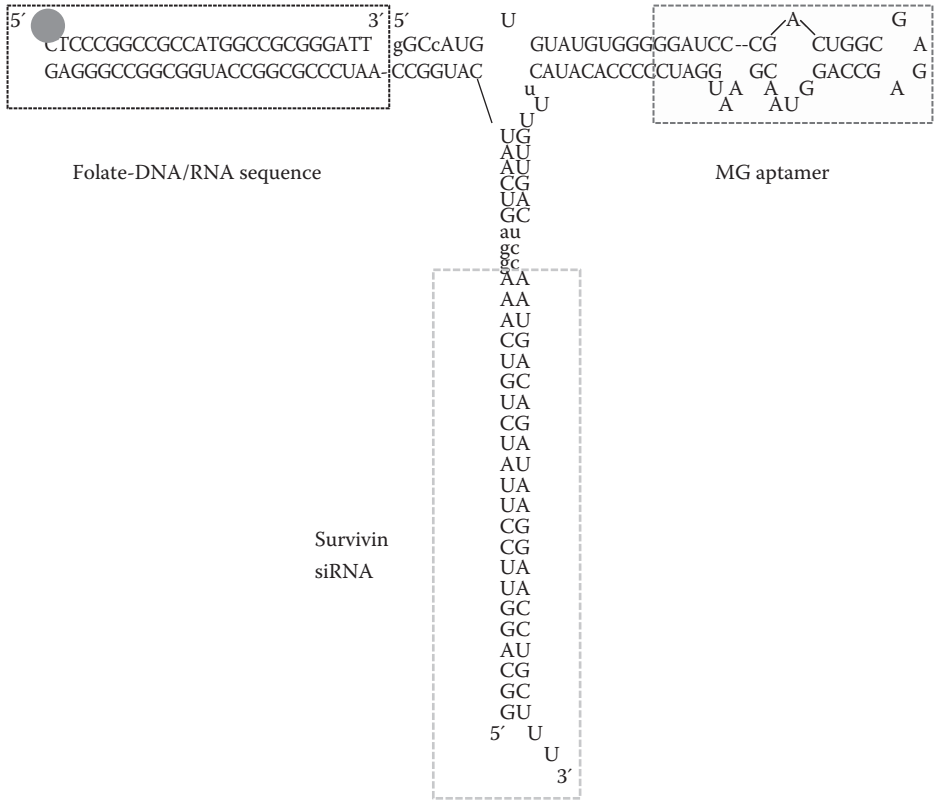


**FIGURE 19.5**
Diagram of RNA nanoparticle harboring malachite green aptamer, survivin siRNA and folate-DNA/RNA sequence for targeting delivery, using 3WJ-pRNA as scaffolds. (Reprinted by permission from Macmillan Publishers Ltd. *Nat. Nanotechnol*, Shu, D. et al., Thermodynamically stable RNA three-way junction for constructing multifunctional nanoparticles for delivery of therapeutics, 6, 658–667, copyright 2011.)

(Figure 19.5; Shu et al. 2011). The sequence for the malachite green aptamer nanoparticle was rationally designed with sequences of three pieces of 3WJ-pRNA motif. These three pieces of RNA strands were synthesized in vitro by transcription from a DNA template with T7 RNA polymerase, and the RNA nanoparticles were then self-assembled when the three RNA pieces were mixed in equal molar ratios. If there is currently no known RNA aptamer available for the desired applications, another approach for the conjugation of aptamers to RNA nanoparticle is to conjugate random sequences to a defined RNA nanoparticle structure. SELEX experiments, as described previously, will then be carried out with this library of random sequence bearing RNA nanoparticles against target peptides, proteins, or cells. The DNA library with random sequences is transcribed into an RNA library, and then partition techniques, such as nitrocellulose partitioning, capillary electrophoresis partitioning, etc., can be utilized to separate the bound and unbound RNA. The bound RNA nanoparticles are further eluted out and used as a template for reverse transcription and PCR for the next round of SELEX experiments.

### 19.3.3 Key Factors for Conjugating Aptamer to RNA Nanoparticles

When designing the aptamer-conjugated RNA nanoparticles, one key factor that needs to be considered is that the aptamer should be conjugated to the outsphere site of RNA nanoparticles, thus the aptamer-targeting delivery functionality can be achieved. Another key factor is to ensure that the aptamer will still fold correctly after conjugation to RNA nanoparticle nucleotides. If the aptamer has a double strand helix RNA end in its structure, we can connect both ends of aptamer to the open helix ends of RNA nanoparticles. If the aptamer has a single strand RNA loop end in its structure, then we can link only one end of the aptamer to the open ends of RNA nanoparticle carriers. For example, when designing anti-gp120 aptamer–conjugated pRNA nanoparticles, Zhou et al. designed two different chimeric pRNA/anti-gp120 aptamer constructs. In one structure, both the double strand helix ends of anti-gp120 aptamer were linked to the pRNA end bases of 23 to 97 (pRNA-A1-D3); in another structure, the anti-gp120 aptamer was directly appended to the 5′-end of pRNA (pRNA-A1-D4; Figure 19.6; Zhou et al. 2011). In vitro studies demonstrated that

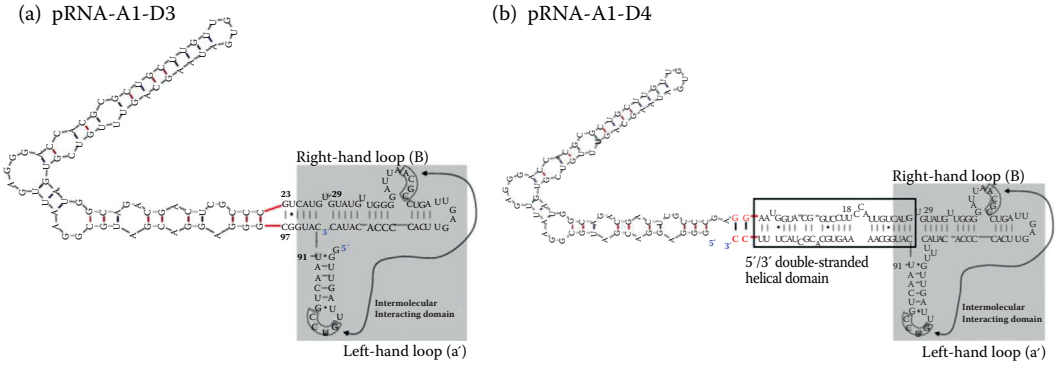


**FIGURE 19.6**
Schematic of pRNA nanoparticle harboring anti-HIV gp120 aptamer. (a) pRNA–A1-D3, the aptamer sequence was inserted into the 3′/5′ double helical domain (23 nt fragment) and loop domain (97 nt fragment). (b) pRNA-A1-D4, the aptamer sequence was directly appended to the 5′ end of pRNA 5′/3′ double-stranded helical domain. (Reprinted from *Methods*, 54, Zhou et al., Dual functional RNA nanoparticles containing phi29 motor pRNA and anti-gp120 aptamer for cell-type specific delivery and HIV-1 inhibition, 284–294, Copyright 2011, with permission from Elsevier.)

both pRNA–aptamer chimeras can specifically bind to and become internalized into cells expressing human immunodeficiency virus (HIV) gp120 with the dissociation constant ($K_d$) being approximately 48 nM for the pRNA-A1-D3, whereas it is approximately 79 nM for the pRNA-A1-D4.

## 19.4 Application Status of RNA Aptamer-Conjugated pRNA Nanoparticles

Because pRNA nanoparticles are composed entirely of RNA, conjugating RNA aptamers to pRNA to form a targeted delivery system will allow all the advantages of RNAs as therapeutic agents to be retained. We have described the structure and synthesis of both pRNA and the aptamers, and discussed the approaches and key factors in the designing of RNA aptamer-conjugated pRNA. Another key obstacle in applying the RNA aptamer–pRNA system for targeted therapy is that RNA is not stable and is susceptible to quick degradation in vivo in the bloodstream. Recently, researchers from Dr. Guo's laboratory found that 2′-F modification of pRNAs are both chemically and metabolically stable in vivo in animals. Importantly, they have shown that pRNA's function and biological activity stays intact, despite this 2′-F modification. To date, this pRNA nanoparticle delivery system has been used to conjugate CD4 aptamers and anti-gp120 aptamers, and was tested in anticancer and viral infection therapies.

Dr. Guo's laboratory has used the pRNA dimer nanoparticle to specifically deliver siRNA against the prosurvival gene called survivin to CD4-positive cells. This is accomplished by replacing the 3′/5′ double helix loop of the pRNA sequence with the survivin-silencing siRNA and by conjugating an anti-CD4 aptamer to the pRNA. They found that this dimer is able to specifically target CD4-positive lymphocytes to silence the target gene expression and reduce cell viability (Guo et al. 2005). Most recently, researchers from Dr. Guo's laboratory have discovered that the 3WJ of pRNA discussed above is the most stable structure found among the 25 3WJ motifs obtained from different biological systems (Shu et al. 2011). They have shown that each arm of the 3WJ-pRNA can carry the abovementioned CD4 receptor–binding RNA aptamer, siRNA, or ribozyme, and bring them into target cells both in vitro and in vivo. Importantly, they have further gone on to show that the 2′-F RNase-resistant form of 3WJ-pRNA also retains its folding and can carry these incorporated functional moieties to target cells both in vitro and in vivo.

Additionally, anti-gp120 aptamers have also been conjugated with the pRNA system by Dr. Rossi's group in their research against HIV-1 infections (Zhou et al. 2008, 2009, 2011; Zhou and Rossi 2011). The HIV-1 virus express a surface protein called glycoprotein gp120, which recognizes the CD4 cell receptor on the host's cells and initiates the membrane fusion that leads to subsequent delivery of viral RNA and enzymes. Once infected by HIV-1, these cells will then also express gp120 on their cell surface. Zhou et al. have previously generated gp120 aptamer chimeras with siRNA targeting the HIV-1 tat/rev gene region and found that these chimeras can be specifically internalized into cells expressing gp120 to silence the expression of target gene. Most recently, Dr. Rossi's group further used the pRNA system developed by Dr. Guo to generate dual functional RNA nanoparticle chimeras with anti-gp120 aptamers, as described previously, and achieved both cell type–specific delivery and targeted inhibition of viral replications (Zhou et al. 2011).

## 19.5 Conclusions and Future Perspective

The high affinity and specificity of RNA aptamers rivaling those of antibodies makes them a promising tool for targeted delivery of therapeutics. As discussed in this chapter, conjugating RNA aptamers to pRNA nanoparticles for targeted therapy has shown great promise in the treatment of cancer and viral infections. In addition to the abovementioned pRNA nanodelivery system, there have also been developments in other approaches for delivering therapeutics using RNA aptamers for disease treatments. With their many advantages as a key component of RNA nanotechnology, including its small size, high stability, multiconjugation capability, and especially its nonimmunogenic nature, RNA aptamers will no doubt find more applications in the targeted therapy arena, especially with more and more RNA aptamers isolated against an ever-increasing repertoire of disease targets. With strong interest and further development of RNA nanotechnology, and with the recent approval of RNA as a therapeutic by the Food and Drug Administration, we should also expect a bright future for RNA aptamers not only as a delivery tool for targeted therapy but also beyond.

## Acknowledgments

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*Review Article*

# RNA aptamers and their therapeutic and diagnostic applications

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**Abstract:** RNA Aptamers refer to RNA oligonulceotides that are capable of binding to specific targets with high affinity and specificity. Through a process called Systematic Evolution of Ligands by EXponential enrichment (SELEX), a number of RNA aptamers have been identified against various targets including organic compounds, nucleotides, proteins and even whole cells and organisms. RNA aptamers have proven to be of high therapeutic and diagnostic value with recent FDA approval of the first aptamer drug and additional ones in the clinical pipelines. It has also been found to be a particularly useful tool for cell-type specific delivery of other RNA therapeutics like siRNA. All these establish RNA aptamers as one of the pivotal tools of the emerging RNA nanotechnology field in the fight against human diseases including cancer, viral infections and other diseases. This article summarizes the current advancement in the identification of RNA aptamers and also provides some examples of their therapeutic and diagnostic applications.

**Keywords:** RNA nanotechnology, RNA aptamer, SELEX, RNA therapy, drug delivery

## Introduction

RNA Aptamers are defined as RNA oligonucleotides that bind to a specific target with high affinity and specificity, similarly to how an antibody binds to an antigen. Isolation of aptamers from randomized pools of RNA by using a method called Systematic Evolution of Ligands by EXponential enrichment (SELEX) was first developed by Gold and Turek, and by Ellington and Szostak [1, 2]. These RNA molecules were termed as "aptamers," with etymology stemming from the Greek word *aptus*, which means "to fit" [1, 3]. To date, various aptamers have been successfully selected against different targets and have begun to show promise as diagnostic, prognostic and therapeutic tools in a wide-range of applications including the treatment for human diseases such as cancer, viral infection and macular degeneration [4-11].

Although this review article focuses on the development and applications of RNA aptamers, it is important to note that aptamers can also be made of DNA and protein as well. However, there are numerous advantages to RNA aptamers as a pivotal tool of RNA nanotechnology when compared to DNA aptamers, protein aptamers, and antibodies [4, 6, 9, 12]. First, although RNA aptamers function similarly to antibodies, they are known to have low or no immunogenicity when compared to other macromolecules such as proteins. A second advantage to RNA aptamers is that when compared to their peptide and antibody counterparts, they are easier to synthesize in large quantities in a controlled manner, and achieve defined structure and stoichiometry. Furthermore, nucleic acids such as RNAs are generally considered to be more thermodynamically stable than peptides or antibodies. Importantly, RNA aptamers can be further chemically modified (e.g. 2'deoxy, 2'F, 2'NH3, 2'OMe), which has been found to greatly improve their stability in the blood stream and resistance to RNAase shearing. Moreover, RNA aptamers are single-stranded in nature, which allows for a unique tertiary structure and leads to tighter and more specific binding. The single-stranded composition of RNA aptamers also makes them smaller in size and easier to enter into cells than DNA aptamers of the same length in nucleotides. Importantly,

this size-advantage and easy conjugation feature of RNA aptamers also aid their ability to carry additional ligands for specific targeting or therapeutic agents for intracellular drug delivery.

## SELEX: systematic evolution of ligands by exponential enrichment

Aptamers are chemically synthesized and selected for their high affinity and specificity for a certain target through the SELEX process [1-3]. Generally speaking, an RNA aptamer is about 56-120 nucleotides long and is comprised of a variable region and a constant region. The variable portion of the aptamer is located in the center and ranges from 20-80 nucleotides in length. Constant nucleotide sequences can be found on both sides of the variable region (the 5' and 3' ends) with about 18-20 nucleotides in length. To date, through traditional basic SELEX and variations of this approach such as Cell-SELEX, Cross-Over SELEX and Tissue-SELEX (**Figure 1**), a good number of RNA aptamers have been isolated with the capability of binding numerous targets, including small molecules, proteins, cells, and even organisms [5-7, 9].

### *The basic SELEX procedure*

When initiating a SELEX procedure to isolate RNA aptamers, a library of RNA oligonucleotides with a complexity upwards of $10^{14}$ is first generated wherein the sequence of nucleotides in the center region is randomly generated and the flanking segments are constant, as described above [1-3, 5, 7, 10]. These oligonucleotides are then exposed to the intended target of interest under the desired conditions. A subset of the oligos will bind to the target, and are therefore potentially the desired aptamer. They are first partitioned from those that do not bind; reverse transcribed, amplified using PCR and further transcribed to generate a new pool of RNA oligonucleotides. Subsequently, this pool of binding oligonucleotides is exposed to the target for a second time, and once again the binding oligonucleotides are isolated and amplified. This sequence is repeated, usually 5-15 times, in order to isolate the aptamers that have the best binding properties. To ensure that the aptamer binds *only* the desired target, oligos that bind the non-desired target are often removed from the pool of aptamers through a process called "negative selection," which uses targets that are similar, but not identical, in structure. In this fashion, the aptamer that binds most specifically and sensitively to the desired target can be discovered and amplified. Typically, aptamers selected through the SELEX process have dissociation constants (kd's) with the target ranging from high pico-molar to low nano-molar.

When done manually, the SELEX procedure can take more than a month to complete. Cox *et al.* recognized the potential for improving the speed of the procedure by automation. By coupling use of a robot and specially designed software, they were able to complete upwards of 10 rounds of SELEX per day [13]. This not only made it possible to complete a SELEX experiment in a matter of days, but also improved the consistency of results.

### *Cell-SELEX*

There are also different variations of the SELEX procedure that can be done; one of which is called Cell-SELEX [5, 7, 10]. The fundamental difference of this procedure from traditional SELEX is that whole living cells are used as targets. Cell-SELEX has been most widely used to isolate RNA aptamers against known or novel cell surface markers of human diseases for therapy and biomarker discovery. Cell-SELEX offers the advantage of being able to create aptamers against a desired cell type, even if the cell's markers are not known. All cells are known to express certain protein markers on their surface. In a disease state such as cancer, the levels of some these markers can increase, additional modifications may be added, and completely new cell markers can appear on the cell's surface, all of which can be used to differentiate these diseased cells from their normal state. Through this approach, RNA aptamers have been isolated against cell surface markers such as T-cell acute lymphoblastic lymphoma, glioblastoma, and small cell lung cancer, among others.

Cell-SELEX, however, is not without its drawbacks. The cells can be damaged and even undergo cell death during the procedure to separate the bound aptamers from the unbound. These dead cells bind aptamers non-specifically and therefore supply a pool of aptamers with decreased specificity for the desired protein target. Currently, work is being done to separate the cells that are live from those which

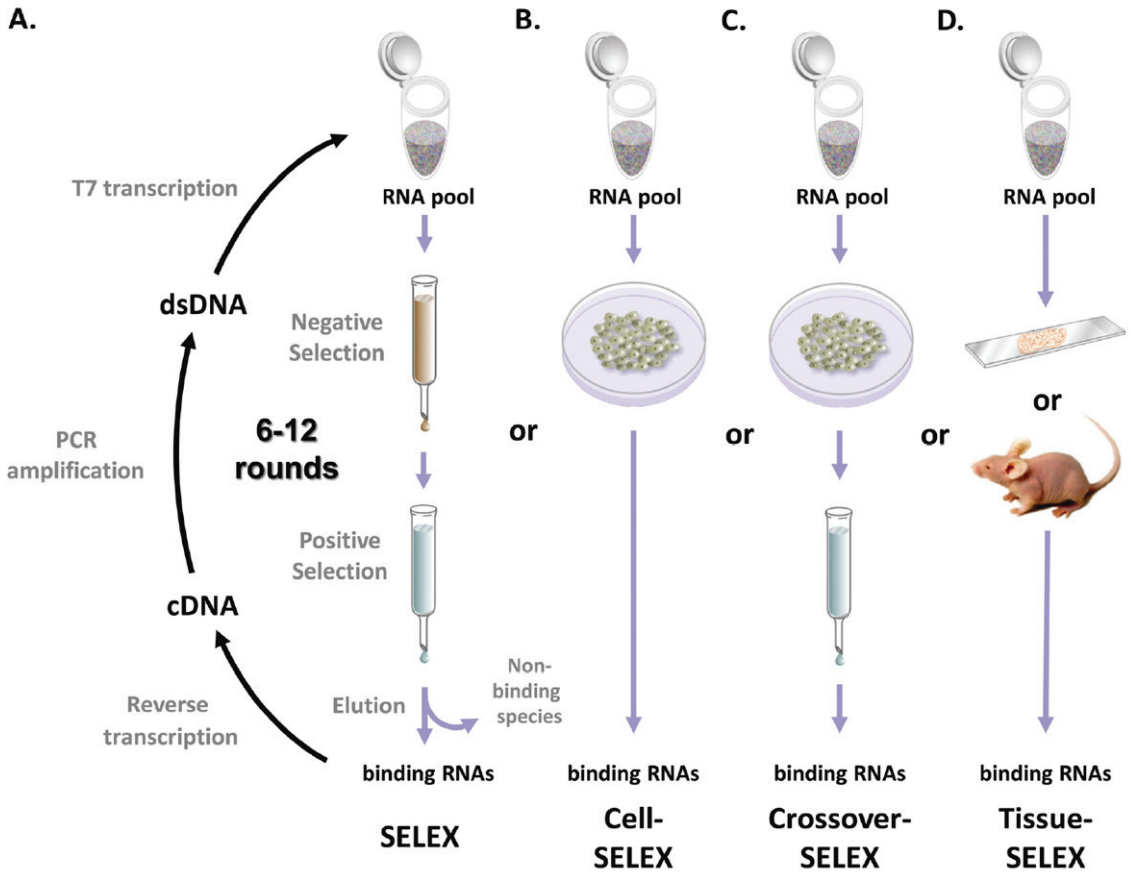


**Figure 1.** An overview of the SELEX procedures. A. SELEX: A RNA pool undergoes negative and positive selection (gray arrows), followed by reverse transcription, PCR amplification, and T7 transcription (black arrows). B. Cell-SELEX, C) Crossover-SELEX, and D) Tissue SELEX instead use B) whole cells, C) whole cells plus protein markers, or C) tissue sections or live animals for the selection step(s), followed by the steps outlined by the black arrows as in A) All of the above procedures are usually repeated for 6-12 cycles.

have died or been damaged by using the techniques such as high-speed fluorescence-activated sorting (FACS) to separate these two populations of cells. Adding to the problem of non-specific binding is the fact that there are multiple cell markers on the cells. Because of this, aptamers may bind to the other cell membrane proteins and therefore still pass through the SELEX process. As such, it becomes very important to do the negative selection steps, as described above and below, in order to filter these aptamers out of the pool. This ultimately requires more rounds of SELEX to be carried out and thus decreases the efficiency of the process [5].

*Cross-over and tissue-SELEX*

In light of the above multiple cell markers issue in Cell-SELEX, Cross-over SELEX has recently been developed for situations in which cell markers are already known for the disease process of interest. In this method, the initial rounds of SELEX are carried out using whole cells as targets as in Cell-SELEX, while later rounds are accomplished using purified cell marker protein targets. In this way, the investigator gains the advantages of both processes and yet diminishes the disadvantage of interference from other proteins [5, 7, 10].

Another variation on SELEX is called Tissue-SELEX. In this process, researchers are able to, for example, take a section of a tumor tissue and isolate aptamers that may bind different components of the tumor, including the extracellular matrix, cellular membranes and intracellular components [5]. This process is accomplished by exposing diseased tissue section slides to the pool of aptamers. The healthy tis-

sues are also exposed to serve as a control so the investigator can identify aptamers that bind solely to the diseased tissue. Remarkably, this tissue-SELEX strategy has recently been expanded to in vivo applications by using tumor-bearing mice to identify RNA aptmaers with the ability to recognize cell markers for diseases such as hepatic colon cancer metastases [14].

## Thearpeutic and diagnostic applications of RNA aptamers

Through the above mentioned various SELEX approaches, dozens of RNA aptamers have been isolated against mostly cell surface markers, but also for intra- and extracellular components of key signaling pathways (**Table 1**) [4, 6, 7, 9]. The high binding affinity and specificity of RNA aptamers, among and other characteristics, make them highly attractive for therapeutic and diagnostic applications to target these markers or signaling pathways. In fact, this area of research has recently gained huge momentum with FDA's approval of the first RNA aptamer-based therapeutics for clinical use. Here, we will summarize the current advancements and provide several examples of applying RNA aptamers for targeted therapy, as well as their diagnostic applications in disease diagnosis, imaging, and new biomarker discovery.

### *Therapeutic applications of RNA aptamers*

Therapeutic potentials of RNAs, including ribozymes, short hairpin RNA (shRNA), siRNA, miRNA, antisense oligonucleotides (AS OGNs) and RNA aptamers, has long been extensively studied [4, 6, 7, 10, 12]. RNA aptamers have unique advantages because, in addition to their intracellular targeting capability, they can directly bind to extracellular targets to inhibit, and in some cases activate their functions, whereas other RNA-based therapeutics must first enter the cell to carry out their functions. In addition, by taking advantage of their ability to bind cell surface proteins, RNA aptamers have also been found to be particularly powerful tools to deliver a variety of therapeutic agents such as small molecules, peptides, and especially RNA-based therapeutics into specific cell types for the treatment of human diseases. In this section, we will provide several examples for recent advances using RNA aptamers as thereaputic agents and as cell-specific delivery tools for RNA-based therapy.

### *RNA aptamers as therapeutic agents*

With the advancement of RNA nanotechnology and development of RNA aptamers suitable for systematic delivery, RNA aptamers have become attractive thereaputic agents against many targets, especially those in the ocular compartments, blood stream and cell surface proteins [4, 6]. In 2004, Pegaptanib (Macugen, Eyetech Pharmaceutics/Pfizer), an aptamer against Vascular Endothelial Growth Factor (VEGF), become the first RNA aptamer approved by US Food and Drug Administration for therapeutic use against age-related macular degeneration (AMD) [15]. Currently, there are also several other RNA aptamers under clinical and preclinical trials for the treatment of diseases such as diabetes and cancer. Here, we will briefly introduce Pegaptanib and also include an update on current efforts to identify and characterize RNA aptamers against the epidermal growth factor receptor (EGFR) family members that are often overexpressed in cancers as examples for future therapeutic applications of RNA aptamers.

*Vascular endothelial growth factor:* Vascular Endothelial Growth Factor (VEGF) is a protein that plays essential roles in both physiologic and pathologic angiogenesis [16-19]. VEGF acts to promote angiogenesis through binding to its receptors (receptor tyrosine kinsases VEGFR1 and VEGFR2) to activate its downstream signaling pathways. Through alternative splicing, the VEGF gene expresses 4 major isoforms of 121, 165, 189 and 206 amino acids, respectively. $VEGF_{165}$ isoform has been found to be solely responsible for the abhorrent neovascularization in age-related macular degeneration (AMD) and diabetic macular edema (DME) [15]. Taking advantage of that, researchers have carried out studies to isolate the RNA aptamer that specifically targets the $VEGF_{165}$ isoform [20-22]. In these studies, they identified several anti-VEGF aptamers with very high affinity and specificity for the $VEGF_{165}$ isoform. Further modifications were extensively examined to increase its stability in serum by fluorination, methylation, and addition of a 3'-3'-linked deoxythmidine terminal cap as well as a 5' polyethylene glycol moiety [15]. Preclinical experiments identified one of these aptamers that has the highest biological activity in inhibiting $VEGF_{165}$'s functions as an endothelial mitogen and vascular permeability

**Table 1.** A list of RNA aptamer sequences and their targets

| Aptamer target | Aptamer sequence (5' → 3') | Disease Target/ Applications | Ref. |
|---|---|---|---|
| 4,4'-methylenedianiline (MDA) | CUGCGAUCAGGGGUAAAUUUCCGCGCAGGCUCCACGCCGC | MDA is an aromatic carcinogen | [61] |
| Acetylcholine receptor (AChR) | GCUAGUAGCCUCAGCAGCAUAGUUUCGCCGCUAUGCAGUA | Neuromuscular disorder | [62] |
| African trypanosomes | AUCGCUACUGCGCCGGUUGCGGCUUGCGGUUGCAACGCCA | Chagas' disease | [63] |
| AMPA receptor GluR2Qflip | GGGCGAAUUCAACUGCCAUCUAGGCAGUAACCAGGAGUUAGUAGGACAAGUUUCGUCC | Cerebral ischemia, Atrophic Lateral Sclerosis (ALS) | [64] |
| Beta Secretace (S10) | GGGAUAGGAUCCACAUCUACGUAUUAGUACACGUCGGCCACCUACGCGAAGUG GAAGCCUCAUUUGUUCACUGCAGACUUGACGAAGCUU | Alzheimer's Disease | [65] |
| Beta Secretase (TH14) | GGGAUAGGAUCCACAUCUACGUAUUACGCAACGCCGGGCCACUACGCGAAUGGC AAGCCCGUCGACUUCACUGCAGACUUGACGAAGCUU | Alzheimer's disease | [65] |
| CD4 | CUCAGACAGAGCAGAAACGACAGUUCAAGCCGAA | HIV | [46] |
| CTLA-4 | GGGAGAGAGGAAGAGGGAUGGGCCGACGUGCCGCAACUUCAACCCUGCACAACCAAUCCGCCCAUAACCCAGAGGUCGAUAGUACUGGAUCCCCCC | Cancer | [66] |
| EGFR (E07) | UGCCGCUAUAAUGCACGGAUUUAAUCGCCGUAGAAAAGCAUGUCAAAGCCG | Cancer | [30] |
| EGFR (J18) | GGCGCUCCGACCUUAGUCUCUGCAAGAUAAACCGUGCUAUUGACCACCCUCA ACACACUUAUUUAAUGUAUUGAACGGACCUACGAACCGUGUAGCACAGCAGA | Cancer | [29, 67] |
| EGFRvIII (E17) | ACCAAAAUCAACGCAAAGAGCGCGCCUGCACGUCACCUCA | Cancer | [68] |
| Erythrocyte membrane protein 1 (PfEMP1) | GGGAAUUCGACCUCGGUACCAACAAUACGACUACACCAUCAAAAGUAUUAUCUUGCAUCGAAGGUUGGCGUAGCAAGCUCUGCAGUCG | Malaria | [53] |
| gp120 | GGGAGACAAGACUAGACGGUGAAUGUGGGCCACGCCCGAUUUUACGCUUUUACCCGCACGCGAUUGGUUUGUUUCCC | HIV Infection | [69] |
| HER3 | GGGAAUUCCGCGUGUGCCAGCGAAAGUUGCGUAUGGGUCACAUCGCAGGCACAUGUCAUCUGGGCGGUCCGUUCGGGAUCCUC | Breast cancer | [67] |
| Human keratinocyte growth factor | CCCAGGACGAUGCGGUGGUCUCCCAAUUCUAAACUUUCUCCAUCGUAUCUGGG | Cancer, | [70] |
| L-selectin | UAACAACAAUCAAGGCGGGUUCACCGCCCCAGUAUGAGUA | Inflammation, Post-ischemic processes | [71] |
| Neruotensin-1 (NTS-1) | ACAGATACGGAACTACAGAGGTCAATTACGGTGGCCACGC | Neurologic Diseases | [72] |
| NF-κB | CAUACUUGAAACUGUAAGGUUGGCGUAUG | Inflammatory, Cancer | [73] |
| Phosphatidylcholine: cholesterol liposomes | GGGAUCUACACGUGACUGACUUACGAGACUGUCUCGCCAAUUCCAGUGGGCCUGCGGAUCCU | Membrane permeability | [74] |
| PSMA (A10) | GGGAGGACGAUGCGGAUCAGCCAUGUUUACGUCACUCCUUGUCAAUCCUCAUCGGCAGACUCGCCCGA | Prostate cancer | [36] |
| Raf-1 | GGGAGAUCGAAUAAACGCUCAAUUUGCCUCGACGGUCUGCGAAUAGAACGCGAACCGUGAUUAGUGUACAAGGAUUCGGUUUUCGACAUGAGGCCCCUGCAGGGCG | Cancer | [75] |
| RET receptor tyrosine kinase | GCGCGGGAATAGTATGGAAGGATACGTATACCGTGCAATCCAGGGCAACG | Multiple endocrine neoplasia | [76] |
| TCF-1 | GGGGAGCUCGGUACCGGUGCGAUCCCCUGUUUACAUUGCAUGCUAGGACGACGCGCCCGAGCGGGUACCGAUUGUGUCGUCGGAAGCUUUGCAGAGGAUC | Colon cancer | [77] |
| Tenascin-C (AptamerTTA1) | GGGAGGACGCGUCGCCGUAAUGGAUGUUUUGCUCCCUG | Glioblastoma and Breast cancer | [78] |
| TGF-β type III receptor | GGGCCAGGCAGCGAGAGAUAAGCAGAAGAAGUAUGUGACCAUGCUCCAGAGAGCAACUUCACAUGCGUAGCCAAACCGACCACACGCGUCCGAGA | Ovarian cancer | [79] |
| Tumor necrosis factor superfamily member 4-1BB | GGGAAGAGAGGAAGAGGGAUGGGCGACCGAACGUGCCCUUCAAAGCCGUUCACUAACCAGUGGCAUAACCCAGAGGUCGAUAGUACUGGUCCCCCC | Mastocytoma | [80] |
| Tumor necrosis factor superfamily member OX40 | GGGAGGACGATGCGGCAGUCUGCAUCGUAGGAAUCGCCACCGUAUACUUUCCCACCAGACGACUCGCUGAGGAUCCGAGA | Cancer | [81] |
| VEGF | CGGAAUCAGUGAAUGCUUAUACAUCCG | Age related macular degeneration | [82] |
| Wilms tumor protein (WT1) | GAUAUGGUGACCACCCCGGC | Wilms tumor | [83] |
| $\alpha_v\beta_3$ integrin | GGGAGACAAGAAUAAACGCUCAAUUCAACGCUGUGAAGGGCUUAUACGAGCGGAUUACCCUUCGACAGGAGGCUCACAAAAGGC | Anti-cancer, anti-thrombotic, anti-inflammatory | [84] |
| β-catenin | GGACGCGUGGUACCAGGCCGAUCUAUGGACGCUAUAGGCACACCGGAUACUUUAACGAUUGGCUAAGCUUCCGCGGGGAUC | Colon cancer | [85] |

enhancer through a series of in vitro and in vivo experiments. Importantly, this aptamer also exhibited no toxicity and very long half-life in pharmacokinetics studies with one study showing biologically active aptamers still remain in eye 28 days post single intravitereous injection in monkeys [23]. With its great success in preclinical studies, this aptamer was eventually

developed into a drug that would undergo clinical trials for treatment of both AMD and DME. The resultant drug was named "Pegaptanib" and has completed Federal Drug Administration (FDA) trials to become available for treatment of AMD in 2004.

*Epidermal growth factor receptor family:* The epidermal growth factor receptor (EGFR) family consists of four closely related cell membrane receptor tyrosine kinases: EGFR (ErbB-1), HER2/c-neu (ErbB-2), Her 3 (ErbB-3) and Her 4 (ErbB-4) [24]. These EGFR family members can form homodimer and heterodimer to activate their kinase activities and downstream signaling cascades that can be facilitated by ligands such as EGF, TGF-a, heregulin. It has been found that mutations affecting the expression or activity of EGFR family proteins are often associated with the development of a wide variety of types of cancers including breast cancer, glioblastoma and lung cancer, among others [25]. Importantly, blocking their aberrant activation, by monoclonal antibodies (mAb) that target extracelluar dimerization or ligand binding domain or by small molecules that target intracellular kinase domain, could significantly inhibit tumor growth and sensitize the tumors to traditional chemotherapy and radiotherapy [26, 27]. Owing to the above mentioned advantages of RNA as nanotherapeutics, not surprisingly, efforts have been taken by several laboratories to identify RNA aptamers that can specifically bind and block the activation of these EGFR family members.

A30 binding to the oligomeric state of extracellular domain of HER3 is the first RNA aptamer selected against an EGFR family member. It was found that high-affinity binding of A30 inhibits heregulin-dependent activation of the pathway and growth of breast cancer MCF-7 cells [28]. By using purified extracellular domain of human EGFR, Ellington's group has initially identified a predominant RNA aptamer (J-18) with a $K_d$ of about 7 nM [29]. They found this RNA aptamer is able to specifically bind and deliver gold particles into cancer cells expressing EGFR. To further identify RNA aptamers for potential in vivo usage, they performed similar experiments but used more stable 2'-fluoropyrimidine modified RNA aptamer libraries [30]. In this screen, they identified a RNA aptamer (E07) with even higher binding ability ($K_d$ = 2.4 nM). They found that E07 is able to compete with epidermal growth factor (EGF) for binding to EGFR or EGFRvIII, a mutant form of EGFR found on breast and lung cancer, as well as Glioblastoma Multiforme (GBM). It is also worth mentioning that GBM is one of the most common and aggressive malignant brain tumors in adults, but is largely unresponsive to current therapy. In this study, they have shown this aptamer could be a promising candidate for anti-tumor therapy because it is able to not only block EGFR activation but also prevent tumor cell proliferation in 3D culture. HER2 is often amplified and over-expressed in human breast cancer and has proven to be a key therapeutic target for the disease [31]. Most recently, by using HER-2 over-expressing cells and counter selection with these cells pre-treated with HER-2 siRNA and HER-2 negative cells, Kang et al have used the whole cell SELEX approach and isolated several high affinity RNA aptamers. Although further functional tests are still needed, these and above mentioned RNA aptamers against EGFR family members could have important therapeutic and diagnostic implications in the future.

*RNA aptamers as delivery tools for targeted therapy*

Recent isolation of RNA aptamers against cell surface markers of various human diseases has led to the application of these RNA aptamers for targeted delivery of RNA therapeutics, especially those based on RNA interferences (RNAi): small interference RNA (siRNA), short-hairpin RNA (shRNA) or microRNA (miRNA) [32]. RNAi was initially discovered in the nematode worm Caenorhabditis elegans and later confirmed in mammalian cells by using synthetic double stranded small RNA around 20-30 nt in length [33]. Once inside the cytoplasm of the cell, siRNA or shRNA is recognized by a protein complex called RNA-induced silencing complex (RISC), which led to its activation and eventual cleavage of the targeted mRNA, thus preventing its protein production [34, 35]. With RNAi technology, it has become possible to specifically target virtually all desired cellular proteins of any given pathway in spite of its functions or cellular localization. However, despite these promises, siRNAs, shRNAs or miRNAs first need to be delivered into the cytosol of the targeted cells for them to function, which has become one of the major focuses of RNAi-based therapies. In this regard, RNA aptamers have pro-

vided a unique tool not only for the recognition of specific target cell but also for the delivery of siRNAs and shRNAs into these cells.

*Prostate specific membrane antigen:* Prostate Specific Membrane Antigen (PSMA) is a prostate cancer marker with increased expression on the surface of prostate cancer cells and the tumor vascular endothelium, but not normal prostate epithelia [36]. The RNA aptamers (A9 and A10) against PSMA have been isolated and subsequently used for intracellular delivery of therapeutics (siRNAs) in multiple studies [37]. Chu et al have non-covalently linked biotinylated A9 aptamer with siRNA against lamin A/C or GAPDH through streptavidin [38]. They observed successful knockdown of these target genes by the Aptamer:siRNA conjugates in PSMA expressing but not PSMA nonexpressing prostate cancer cells, indicating specific targeting of these particles. McNamara et al conjugated RNA aptamer A10 to siRNAs against cancer survival genes polio-like kinase 1 (*PLK1*) and *BCL2* and tested them on prostate cancer cell growth both in vitro and in vivo [36]. They found that these A10-siRNA chimeras were successfully internalized and processed by Dicer, which led to silencing of the expression of these survival genes and subsequent cell death in vitro [36]. They went on to further inject the A10-Plk1 siRNA and control mutantA10-PIK1 siRNA chimeras into prostate tumors in an in vivo mouse prostate cancer xenograph model. It was found that A10-Plk1 siRNAs, but not mutantA10-PIK1 siRNAs, are able to significantly inhibit the prostate tumor growth and mediate tumor regression. In subsequent work, Dassie et al have incorporated modifications, added 3' overhangs, truncated the aptamer sequences to optimize and enhance chimeras' thermodynamic profile and circulating half life for systemic administration, and observed more pronounced regression of PSMA-expressing tumors in vivo [39].

Most recently, this PSMA aptamer has also been reported to deliver siRNAs to induce tumor immunity in one study, and to enhance ionized radiation sensitivity in another for prostate cancer therapy [40, 41]. In the first study, Pastor et al used A10 aptamer to deliver siRNA against Upf2 and Smg1, key components of the RNA surveillance pathway called non-sense mRNA decay (NMD) pathway [40]. NMD pathway normally functions to prevent mRNAs expression from premature termination. Disruption of this process can lead to generation of antigens recognized by the immune system as foreign and their subsequent immune-mediated rejection. They found that A10 conjugated Upf2 or Smg1 siRNA can be specifically targeted to tumor cells and inhibit their growth in both cell culture and in vivo xenograft studies. Significantly, the ability of A10-Smg1 to inhibit tumor growth was superior to that of vaccination with granulocyte–macrophage colony-stimulating factor (GM-CSF)-expressing irradiated tumor cells, and could be further enhanced by co-stimulation. In the second study, Ni et al used A10 aptamer-shRNA chimera to target DNA-activated protein kinase (DNA-PK) to increase the radiosensitivity of prostate cancer cells expressing PSMA [41]. It is well known that ionizing radiation causes damage to cell DNA such as inducing breaks in the double stranded DNA or changes in the DNA sequence. Although ionizing radiation has proven to be a powerful tool in cancer therapy, it is highly nonspecific and also damages normal cells. It is also known that cells have developed ways to correct these DNA damage insults to its genome by means such as double-strand break repair and excision repair. Therefore, if the cells are incapable of repairing the damage induced by radiation, they will likely lose their ability to withstand the radiation and will require less radiation for their treatments. Ni et al carried out a siRNA library high throughput screen to identify the key genes responsible for the cell's repair mechanisms in PSMA expressing prostate cancer cells and found DNA-PK as one of such target. They found that A10-3 aptamer-DNA-PK shRNA can specifically reduce DNAPK in prostate cancer cells in vitro, in vivo in xenograft models, and even in human prostate tissues. It has also been shown that intravenous administration of A10-3-DNAPK shRNA chimeras greatly sensitized the PSMA positive prostate tumor to ionized radiation. Importantly, it allows the levels of radiation necessary to treat the cancer to be low enough to significantly decrease the damage to surrounding tissues such as the bladder and rectum.

*CD4 aptamer and pRNA nanodelivery system:* Although RNA-based therapy has become a promising avenue for the treatment of many human diseases, a major challenge that remains is the systemic and intracellular delivery of these moieties (siRNA, ribozyme etc) to

the desired target cells. RNA aptamers have provided a unique capability for specific cellular targeting but their sub-nano size, even after conjugation to these RNA therapeutics, subjects them to body clearance. In this regard, the pRNA nanoparticle delivery system pioneered by Dr. Guo provided an ideal method for nanoscale delivery suitable for in vivo delivery [12]. Since pRNA nanoparticles are composed of all RNA, it is the most natural choice for the delivery of RNA therapeutics because using an all RNA delivery system will allow all the advantages of RNAs as therapeutic agents to be retained.

pRNA monomer is a 117-nt long RNA molecule about 11nm in size and constitutes one of the six subunits of DNA packaging motor of bacteriophage phi29. pRNA has two functional domains that can fold independently: DNA translocation domain and prohead binding domain. The DNA translocation domain is composed of a 3'/5' double helix loop while left- and right- hand loops form the prohead binding domain. By replacing the 3'/5' double helix loop of the pRNA sequence with siRNA against a pro-survival gene called survivin, Dr. Guo and colleagues have first demonstrated that it can fold properly to knockdown the expression of target genes and inhibit tumor growth both in vitro and in vivo. One important feature of this pRNA system is its ability to form pRNA dimers and multimers through right-hand loop (A) and left hand loop (B) interactions. Taking advantage of that, Dr. Guo and colleagues generated pRNA dimers with one pRNA(a'-B) carrying RNA aptamer against CD4 receptor and the other pRNA(A-b') conjugated with siRNA against survivin. They found this dimer is able to specifically target CD4 positive lymphocytes to silence the target gene expression and reduce cell viability [42].

Another key obstacle for RNA-based therapy is that RNA is susceptible to quick degradation in the blood stream during in vivo delivery. To overcome that, Dr. Guo's laboratory has developed a RNAse-resistant form of pRNA by 2'-deoxy-2'-fluoro (2'-F) modification at the ribose rings of C and U [43]. They found that 2'-F modified pRNAs are highly stable and can fold into its 3-D structure properly. Most importantly, they have shown that this 2'-F modification of pRNA does not alter its functions and is fully biologically active. This RNAse-resistant phi29 pRNA has also recently been tested for pharmacological characteristics in systemic delivery [44]. It was found that 2'-F-modified pRNA nanopaticles are very stable chemically and metabolically in vivo in mice. Most recently, Dr. Guo's laboratory has discovered that the three way junction (3WJ) of pRNA that connects its left hand loop, right hand loop and helix domain can form a complex that is the most stable structure found among 25 3WJ motifs obtained from different biological systems [45]. They have shown that each arm of the 3WJ-pRNA can carry the above mentioned CD4 receptor binding RNA aptamer, siRNA or ribozyme and bring them into target cells both in vitro and in vivo. Importantly, they have further gone on to show that the 2'-F RNAse resistant form of 3WJ-pRNA also retains its folding and can carry these incorporated functional moities to target cells both in vitro and in vivo.

*gp120:* John Rossi's group used a RNA aptamer against gp120 for targeted delivery of siRNA in their fight against Human Immunodeficiency Virus (HIV) infections [46-49]. When a person is exposed to and infected with HIV-1, the virus enters the individual's body and targets cells that express the CD4 receptor. The HIV-1 virus uses its surface protein called glycoprotein gp120 to recognize the CD4 cell receptor and initiate the membrane fusion and subsequent delivery of viral RNA and enzymes. Once infected, these cells will then also express gp120 on their cell surface. Zhou et al generated gp120 aptamer chimeras with siRNA targeting the HIV-1 tat/rev region. They found the chimera can be specifically internalized into cells expressing gp120 to silence the target gene expression. Importantly, these gp120 aptamer-siRNA chimeras exhibited potent and lasting effect on inhibiting HIV replication in T cells without triggering interferon response. Rossi's group recently used the same pRNA system developed by Dr. Guo, as described above, to generate dual functional RNA nanoparticles and also achieved both cell type specific delivery and targeted inhibition [49]. In this study, they used pRNA(a'-B) to form a chimera with RNA aptamer that recognize gp120 and linked siRNA against HIV-1 tat/rev to the complementary pRNA(A-b'). They found the pRNA(a'-B)-aptamer chimera could specifically bind to HIV infected cells and that pRNA(A-b')-siRNA chimera could be processed by Dicer as expected. They further reported that incubating the

pRNA(a'-B)-aptamer/pRNA(A-b')-siRNA dimers with HIV infected cells led to successful binding by the aptamer and delivery of the siRNA. They have also applied 2'-F modified pyrimidines in the sense strand of pRNA-siRNA chimera and found the chimera to be more stable in the serum. Importantly, the modified chimera can still be functionally processed by Dicer to specifically silence the target gene expression, thus paving the way for future in vivo systemic delivery studies.

*Diagnostic applications of RNA aptamers*

Use of RNA aptamers has already been successful in treating AMD and has shown promise in treating prostate cancer, HIV infection and other diseases, while further research will undoubtedly be done to target more cell components for disease treatment. Importantly, in addition to these therapeutic applications, RNA aptamers have also started to play increasingly important roles not only in environmental and food analysis, but also in human disease diagnosis [5, 8, 11, 50]. In this section, we will focus on the role of RNA aptamers in disease diagnosis through imaging, disease cell detection and novel biomarker discovery.

*RNA aptamer as an imaging tool*

Antibodies have long been the focus of such diagnostic studies because of their ability to target certain cell markers. With the development of RNA nanotechnology and the SELEX procedure, RNA aptamers have become a more attractive option when compared to their antibody counterparts. RNA aptamers used for imaging have the same advantages of those used for therapy: low immunogenicity, smaller size, and relatively short time to develop the desired aptamer [51]. Imaging via selective delivery of radionucleotides to tumors has been investigated for many years. When attaching these radioactive substances to aptamers and antibodies, the residual radioactive aptamers in the bloodstream are quickly cleared and excreted, owing to the rapid uptake of Aptamers by tumors. Not only does this allow for superior tumor imaging, but it also decreases the toxicity to normal tissues that was often seen with radioactively labeled antibodies due to their slow clearance from the body [51].

One such example of imaging applications is the RNA aptamer for tenascin-C, an extracellular matrix protein upregulated in a number of tumors such as breast, lung, colon, prostate, glioblastoma, and lymphoma [5, 7]. An anti-TN-C aptamer, TTA1, was discovered, purified, and then modified to increase stability in serum and to allow for subsequent radionucleotide conjugation. Researchers have used radionucleotide $^{99m}$Tc to image the cells that are able to bind to and internalize the TTA1 aptamer. They found TTA1-$^{99m}$Tc exhibited high "signal to noise ratio" as it was quickly uptaken by the tumor cell and eliminated from the bloodstream. Importantly, the tumor retention time of TTA1-$^{99m}$Tc was sufficiently long that the tumor image was much clearer compared to background when imaged using single-photon emission-computed tomography [7]. Another example of RNA aptamers that have potential for tumor imaging in humans is the above mentioned A10 aptamer against prostate cancer marker PMSA. A10 aptamer has been tested for tumor imaging after conjugation with quantum dots, as well as thermally cross-linked supraparamagnetic iron oxide nanoparticle (TCL-SPION) [5]. TCL-SPION nanoparticle is often used as a contrast agent in MRI imaging, and has low systemic toxicity. In this case, A10 aptamer allows TCL-SPION to bind specifically to prostate cancer cells expressing PMSA thus enabling the imaging capabilities of TCL-SPION to be localized to the prostate tumor.

*RNA aptamers in disease diagnosis*

In addition to their role as imaging tools, RNA aptamers can also aid in clinical diagnosis of diseases due to their high affinity to bind specific cell markers. Their small size, stable structure and ease of synthesis also add to their attraction to detect human diseases, even before the symptoms become apparent. Importantly, the easy conjugation and labeling features of RNA aptamers also allow them to be combined with other advanced technologies such as microfluidic cell separation, endogenous nucleic acid analysis, nanoparticle based sensing or flow cytometry to maximize their diagnostic functions [8, 11].

Recently, RNA aptamers have become an attractive tool in detecting diseased cells on a histological section and, most importantly, the presence of very low amounts of circulating disease cells in the bloodstream. One such example is to use the above mentioned RNA aptamer

against EGFR to determine the presence or extent of GBM, a deadly disease that is hard to detect. To achieve that, Wan et al first immobilized the aptamer on a chemically modified glass surface and exposed it to the cells in question, either from serum or the tumor margin [52]. The bound cells were then collected and imaged on a neuro-optical microfluidic platform to quantify the disease cells and determine the extent of disease. Through these procedures, they were able to detect primary human GBM cells expressing high levels of EGFR with high sensitivity and specificity. Therefore, this approach could lead to earlier diagnosis of this highly malignant tumor and monitoring of residue disease after the treatment by detecting circulating tumor cells in the serum. In the case of a tumor resection, this would also allow the surgeon to know whether surgical resection margins of the tumor are free from diseased cells.

RNA aptamers have also shown potential for use in conjunction with flow cytometry to detect diseased cells. Li et al have recently tested RNA aptamers against Human EGFR Related 3 (HER3), Tn-C, PSMA, and EGFR for detection of varieties of human cancer cells [53]. They optimized the assay and received strong signals by fluorescent labeling of biotinylated RNA aptamers with streptavidin-phycoerythrin (SA-PE) for flow cytometry [53]. Although the ability of these RNA aptamers to detect different cancer cell varies and optimization will likely be required for each application, they nevertheless show promise for use of RNA aptamers with flow cytometry to detect a wide variety of human cancer cells. However, more work may still need to be done to improve the current technology, especially because relative to normal cells in a blood sample, there are very few diseased cells, making detection of these cells more difficult. To overcome that, it may be necessary to perform additional steps of *ex vivo* amplification of cells before passing them through the flow cytometer. In addition, further work is still needed to ensure single cells pass through the flow cytometer, as the cell clusters often naturally occur and skew the results [54, 55].

Diagnostically, aptamers can also be utilized in a similar manner that antibodies have been used in a two-site binding assay, the most commonly used diagnostic format today. Using this approach, Drolet et al were able to detect serum vascular endothelial growth factor (VEGF) protein, which plays an important role in angiogenesis and has been used as biomarker for breast cancer, lung cancer and colorectal cancer [56]. In this assay, RNA aptamers targeting VEGF are synthesized and labeled with fluorescein. Alkaline phosphatase conjugated Fab antibody fragments directed against fluorescein were then used to detect fluorescein labeled RNA aptamers. Li et al recently used a different approach and were able to detect VEGF at a biologically relevant concentration of 1 pM [57]. They used immobilized RNA aptamer arrays to enrich serum VEGF, followed by signal amplification using horseradish peroxidase (HRP) conjugated antibodies against VEGF and measurements by surface plasmon resonance imaging (SPRI). Although there is still significant work needed to be done before these and above mentioned approaches can be used clinically, it is clear that RNA aptamer technology has the potential to make early detection of disease possible, and with further development, become indispensable to the medical field.

*RNA aptamers in biomarker discovery*

Identifying and characterizing cell markers for diagnosis and treatment purposes have historically been difficult. As mentioned above, there has been a number of RNA aptamers recently isolated that can be used as biomarkers to differentiate between different cell types or distinguish disease cells from normal cells. In addition, RNA aptamers also have a unique advantage in novel biomarker discovery because RNA can be selected through the SELEX procedure without prior knowledge of these cell markers [5, 58]. Current studies combining 2D gel electrophoresis and mass spectrometry have made it relatively easy to identify soluble biomarkers. However, it still requires a significant amount of these biomarkers, often expressed at low levels, to be isolated for identification. In addition, the amphipathic nature of cell membrane proteins has made identification of cell surface markers using this approach insufficient, because the extraction of these cell surface proteins requires detergent that tends to diminish the signal from mass spectrometry [5]. By immobilizing RNA aptamers, several methods have recently been developed that can be used to

enrich both soluble and membrane biomarkers for subsequent mass spectrometry analysis [59, 60].

Remarkably, Mi et al most recently developed a unique approach to isolate RNA aptamers in vivo against tumor cells in living mice and subsequently used selected RNA aptamers to identify the biomarker for hepatic colon cancer metastases. In this study, they intravenously injected 2'fluro-pyrimidine modified RNA aptamer library into an animal model of intrahepatic colorectal cancer metastases, where mice were implanted with hepatic tumor. Liver tumors were then harvested for RNA molecules extraction and amplification. The resulting pool of RNA was then reinjected and the process mention above was repeated for 14 rounds. They found that RNA aptamer 14-16 was able to specifically stain intrahepatic CT26 tumors both in vitro and in vivo. To further identify the tumor specific protein that RNA aptamer 14-16 interacts with, they further immobilized biotinylated RNA apatmers on streptavidin magnetic beads and then incubated with tumor tissue extracts. After standard washing and gel electorphoresis steps, they are able to use peptide-mass fingerprinting and MS/MS peptide fragment ion-matching to determine that RNA 14-16 binds to Ddx5, a p68 RNA helixase that has previously been reported to be overexpressed in colorectal tumors. With these developments, it becomes quite clear that RNA aptamers have great potential in biomarker discovery and, with further developments and refinement, are poised to become a more mainstream approach and lead to more cell-marker targets for disease diagnosis and treatment.

## Conclusions and future perspective

Although antibodies have proven to be a powerful tool not only in diagnosis but also in disease therapeutics, the high affinity and specificity of RNA aptamers rival antibodies and make them a promising tool in diagnostic and therapeutic application, as evidenced by ample examples given above. With their many advantages over antibodies, such as the small size, high stability, ease and consistency of in vitro synthesis, multi-conjugation capability with other moieties (fluorescein, RNA nanoparticles, etc.) and non-immunogenic nature, RNA aptamers will no doubt find more applications that can be used in conjunction with or complement to antibodies in these areas. We should expect more RNA aptamers to be isolated in the near future against an ever increasing repertoire of targets, using these different SELEX approaches with increased speed and efficiency. With the first RNA therapeutics approved by FDA, we should expect more of them to follow. These RNA aptamers could be used either to block key cellular pathways or as a delivery tools for other RNA nanotechnology based therapeutics. With increasing interest and further improvement of RNA aptamers and RNA nanotechnology, we should also expect wider diagnostic applications using RNA aptamers in imaging, disease detection and biomarker discovery in the years to come.

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COMMENTARY

# MiR-155 at the heart of oncogenic pathways

MF Czyzyk-Krzeska[1,2] and X Zhang[1]

MicroRNAs are increasingly being recognized as oncogenes and tumor suppressors in cancer. MicroRNA-155 (miR-155) is an established oncomiR in breast cancer and regulates several pro-oncogenic pathways. In light of this, Chiang's group has discovered a novel pathway regulated by miR-155. MiR-155 directly targets the VHL tumor suppressor and, by doing so, promotes the activity of HIF transcription factors and angiogenesis. This pathway appears to be particularly relevant in triple-negative breast cancer.

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MicroRNAs are small noncoding RNAs that post-transcriptionally regulate gene expression. Canonical miRNA biogenesis begins with transcription of large primary miRs (pri-miRNA), which are often encoded in intronic regions of larger genes. Pri-miRNA transcripts are then processed by the ribonuclease Drosha to 60–100-bp-long stem-loop structures called pre-miRNAs. These are exported to the cytoplasm where another ribonuclease, Dicer, cleaves the stem-loop structures to 21–25-nucleotide-long dsRNAs. The strand showing complementarity with miR-recognition elements of specific mRNAs is recognized by a member of the Argonaute family of proteins, and is thus incorporated into the RNA-induced silencing complex. There are also Dicer-independent mechanisms that generate functional miRs. The main function of miRs is to suppress the translation of target genes, but they can also process mRNAs for cellular decay. Mature miRs have multiple targets, often members of the same regulatory networks, and often operate in regulatory feedback loops. This positions them as fine-tuning modulators of set points in homeostatic processes in normal cells. There is ample evidence that discrete sets of miRs are induced and repressed in different cancers. Indeed, certain miR expression profiles are specific to particular diagnoses and progression patterns, and predictive of responses to treatment. In cancer, aberrations in the levels of specific miRs may have well-defined tumor-suppressing or oncogenic function. Moreover, as a given miR has multiple targets, multiple pro-oncogenic or tumor-suppressing pathways are affected, and these pathways, in turn, regulate the miRs' expression in a feedback-loop mechanism. In this issue of *Oncogene*, Cheng and colleagues[1] identify another novel pro-oncogenic pathway through which miR-155 (miR-155), which has well-established oncogenic properties, promotes the growth of triple-negative breast cancer. They also show that upregulation of this microRNA is associated with metastasis and poor prognosis in triple-negative breast cancer.

MiR-155 is a well-established oncogene, or oncomiR, in several blood and solid cancers, including breast cancer. It is processed from its own gene, *BIC*, which does not encode a protein product. Currently, more than 100 genes are confirmed to be directly targeted by this miR. The expression of miR-155 is augmented in breast cancer and correlates positively with several clinico-pathological markers, high tumor grade, advanced stage and metastases to lymph nodes, whereas it correlates inversely with overall and disease-free survival.[2] Through its targets, miR-155 acts in the middle of oncogenic loops that repress the activity of several tumor suppressors. In addition, its expression is stimulated by pro-oncogenic conditions in tumors such as hypoxia and inflammation (Figure 1). In NMuMG mouse cells, miR-155 expression is induced by transforming growth factor-β through the direct activity of Smad4 transcription factor. This increased miR-155 activity partially accounts for the transforming growth factor-β-induced epithelial–mesenchymal transition, cancer cell migration and invasion, and involves direct targeting of RhoA by miR-155.[3] In many human breast cancer cell lines, expression of miR-155 is also induced by factors that promote tumor inflammation, such as interleukin 6 or interferon γ. In this pathway, miR-155 inhibits SOCS1 and, by doing so, stimulates activity of the JAK2/STAT3 pathway, thus promoting further tumor inflammation and growth.[4] MiR-155 also contributes to the oncogenic metabolism by stimulating glycolysis through induction of a glycolytic enzyme, hexokinase 2. This induction is partially accomplished through induction of STAT3; however, an additional mechanism is also employed. That is, miR-155 directly targets C/EBPβ, a transcription activator of miR-143, which in turn directly inhibits translation of hexokinase 2.[5] Another crucial target of miR-155 is the pro-apoptotic transcription factor FOXO3a.[6] The significance of this regulation in breast cancer is underlined by a negative correlation between miR-155 and FOXO3a in multiple breast cancer cells lines and tumors. Another tumor suppressor repressed by miR-155 is TP53INP1.[7]

In this issue of *Oncogene*, Chiang's group expands on their previous work by identifying a novel pathway that is regulated by miR-155, and by showing that this pathway promotes oncogenesis in triple-negative breast cancer.[1] They demonstrate that miR-155 is highly upregulated in triple-negative breast cancer, and that its levels correlate with poor survival. They have identified a novel direct target of miR-155, VHL. VHL is a tumor suppressor lost in early stages of clear cell renal cell carcinoma. VHL's canonical function is to act as a substrate-recognition component of the E3 ligase complex, which ubiquitylates and targets alpha subunits of HIFs for proteasomal degradation. This targeting is dependent on the hydroxylation by proline hydroxylases (a specialized group of enzymes) of two prolines within the N-terminal, activating domain of hypoxia-inducible factor (HIF)-α. As this hydroxylation requires molecular oxygen, it is inhibited during hypoxia, resulting in accumulation of HIF-αs and induction of HIF activity. Loss of VHL,

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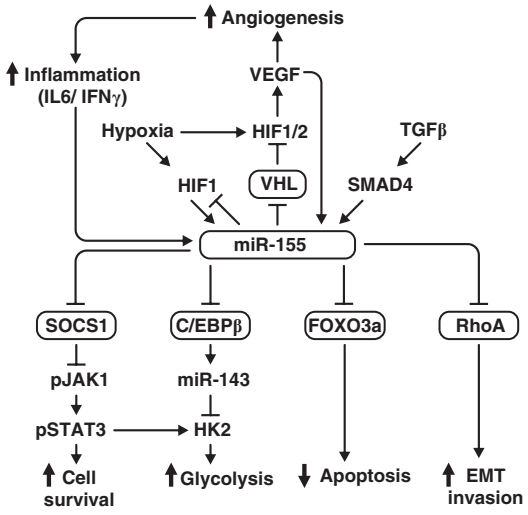


**Figure 1.** Model of the regulatory signaling network upstream and downstream of miR-155 in breast cancer.

such as the genetic loss common in clear cell renal cell carcinoma, mimics the effects of hypoxia. Kong *et al.*[1] reveal a unique mode of regulation for VHL expression in which the direct activity of miR-155 suppresses its translation, and results in increased HIF activity. These data complement the previously published evidence that hypoxia and HIF-1 induce expression of miR-155.[8,9] MiR-155 has a hypoxia-responsive element and its transcription is specifically stimulated by hypoxia-inducible factor 1 (HIF-1), but not HIF-2. Hypoxic induction of miR-155 contributes to the resistance of tumors to radiation therapies.[9] Interestingly, however, miR-155 directly targets HIF-1$\alpha$, but not HIF-2$\alpha$, and suppresses its expression.[8] While on the surface, such activity may seem to contradict the pro-oncogenic functions of miR-155, it is important to note that of the two HIFs, HIF-2 is more oncogenic. Thus miR-155, by repressing HIF-1$\alpha$, may increase the contribution of HIF-2$\alpha$ to the oncogenic process when both HIFs are expressed.

Significantly, Chiang's group performed a detailed analysis of miR-155 expression in clinical breast cancer samples and found that its upregulation was highly correlated with metastasis, poor prognosis and triple-negative breast cancer. Despite recent advancements, breast cancer still remains one of the leading causes of death for women, with more than 1.3 million cases and 450 000 deaths each year worldwide.[10] Breast cancer can be largely divided into four major subtypes: estrogen receptor (ER)-positive, comprising luminal A and luminal B subtypes; HER2 (ERBB2)-amplified; and triple-negative breast cancer.[10,11] In recent years, good strides have been made in the development of various targeted endocrine therapy approaches for treating ER+ and HER2+ breast cancer through endocrine therapies (for example, selective estrogen receptor modulators (SERMs), selective estrogen receptor downregulators (SERDs), aromatase inhibitors (AIs)) and anti-HER2 therapy (for example, Herceptin), respectively. Unfortunately, however, for triple-negative breast cancer that lacks ER and PR expression and HER2 amplification, there is currently no available targeted therapy, leaving treatment by chemotherapy as the only option. Further characterization of the miRs involved in triple-negative breast cancer is critical from the point of view of potential therapeutic applications, such as the use of miR-155 'antagomirs' to target key oncogenic processes including hypoxia and inflammation, to be used alone or in combination with existing chemotherapeutic regimens.

The most recent comprehensive genomic and proteomic analyses of primary breast cancers have revealed unexpected molecular similarity between triple-negative breast cancers and ovarian tumors.[12] These new findings suggest a similar etiology of these cancers, and the potential usage of shared treatment strategies that target common pathways. Interestingly, miR-155 has recently been found to be regulated epigenetically by BRCA1, a common risk factor for both ovarian cancer and triple-negative breast cancer.[10] This discovery placing miR-155 at the heart of the oncogenic pathways could have important therapeutic implications for both of these cancers. Additionally, miR-155 has been reported to be overexpressed in some other cancer types, such as lung and pancreatic cancers. Therefore, it will be of interest to know whether the oncogenic mechanisms promoted by miR-155 discussed above are common to all of these cancers, which may in turn, lead to even broader applications of these findings.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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